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## Antibacterial activity of 2-alkynoic fatty acids against multidrug resistant bacteria

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### Abstract

The first study aimed at determining the structural characteristics needed to prepare antibacterial 2-alkynoic fatty acids (2-AFAs) was accomplished by synthesizing several 2-AFAs and other analogues in 18-76% overall yields. Among all the compounds tested, the 2-hexadecynoic acid (2-HDA) displayed the best overall antibacterial activity against Gram-positive *Staphylococcus aureus* (MIC = 15.6 µg/mL), *Staphylococcus saprophyticus* (MIC = 15.5 µg/mL), and *Bacillus cereus* (MIC = 31.3 µg/mL), as well as against the Gram-negative *Klebsiella pneumoniae* (7.8 µg/mL) and *Pseudomonas aeruginosa* (MIC = 125 µg/mL). In addition, 2-HDA displayed significant antibacterial activity against methicillin-resistant *S. aureus* (MRSA) ATCC 43300 (MIC = 15.6 µg/mL) and clinical isolates of MRSA (MIC = 3.9 µg/mL). No direct relationship was found between the antibacterial activity of 2-AFAs and their critical micelle concentration (CMC) suggesting that the antibacterial properties of these fatty acids are not mediated by micelle formation. It was demonstrated that the presence of a triple bond at C-2 as well as the carboxylic acid moiety in 2-AFAs are important for their antibacterial activity. 2-HDA has the potential to be further evaluated for use in antibacterial formulations.

### Keywords

2-alkynoic fatty acids; antibacterial agents; synthesis; micelles; MRSA; nosocomial infections

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## 1. Introduction

Nosocomial infection is defined as “an *infection acquired in a hospital by a patient who was admitted for a reason other than that infection*” (WHO, 2002). These infections are major source of morbidity and mortality affecting more than 1.7 million patients annually and generating over 99,000 death per year in the United States (Klevens et al., 2007). In addition to the human cost, nosocomial infections affect the quality costs in hospitals as well as the finances of the public health care system (Brachman et al., 1980; Pittet et al., 1994; Stone et al., 2002; Zhan and Miller, 2003). Mechanical ventilation, use of invasive procedures, and patients' immunocompromised status are the principal factors that propitiate nosocomial infections (Inweregbu et al., 2005). This situation is complicated when bacteria gain resistance towards those antibiotics commonly used in hospitals such as penicillins, polyenes, glycopeptides, macrolides, and cephalosporins (Inweregbu et al., 2005). Bacteria develop resistance when they acquire new genetic material as a result of an inadequate antibiotic therapy (Inweregbu et al., 2005; Klevens et al., 2007). Despite the fact that several compounds such as synthetic fluoroquinolones (Bradbury and Pucci, 2008), aminocoumarins (Freitag et al., 2005; Heide, 2009), and cyclothialidines (Kampranis et al., 1999; Rudolph et al., 2001) are being evaluated as antibacterial agents, there is an urgent need to develop new antibiotics to replace those that are not effective.

Among those compounds that are being evaluated as antibacterial agents, unsaturated fatty acids have demonstrated to be biologically active towards Gram-positive bacteria, specifically against *Staphylococcus aureus* (Carballeira et al., 2002; Carballeira et al., 1998; Zheng et al., 2005)<sub>[DS1]</sub>. In the present study, we prepared a series of 2-alkynoic fatty acids (2-AFAs) and other synthetic analogues such as 2-tetrahydropyranyl protected alkynols and 2-alkynols aimed at establishing a structure activity relationship (SAR) with these compounds in order to find the fatty acid with better cytotoxicity against both Gram-positive and Gram-negative bacteria. 2-AFAs are acetylenic fatty acids which have the peculiarity of containing a triple bond (C≡C) at C-2 in their structures. Acetylenic fatty acids have been widely studied by medicinal chemists due to their interesting antimicrobial properties such as antifungal (Carballeira, 2008; Carballeira et al., 2006; Carballeira et al., 2005; Gershon and Shanks, 1978; Li et al., 2003; Li et al., 2008; Xu et al., 2012), antiprotozoal (Carballeira et al., 2012; Tasdemir et al., 2010), and antibacterial activities (Konthikamee et al., 1982; Morbidoni et al., 2006). Acetylenic fatty acids are mainly produced by certain plants as a chemical defense against microorganisms (Cahoon et al., 2003; Carballeira, 2008; Fatope et al., 2000; Li et al., 2003; Li et al., 2008; Xu et al., 2012). Among the acetylenic fatty acids, the 2-hexadecynoic acid (2-HDA) has received the most attention for its antimicrobial and cytotoxic properties (Carballeira et al., 2012; Carballeira et al., 2006; Gershon and Shanks, 1978; Morbidoni et al., 2006; Upreti et al., 1981; Wood and Lee, 1981). For example, Konthikamee *et al.* reported that 2-HDA was particularly active against the Gram-positive cocci, including penicillin-resistant *Staphylococcus aureus*<sub>[DS2]</sub>, at concentrations of 21.4-56.8 μM (Konthikamee et al., 1982). In addition, it was reported that 2-HDA was active against the Gram-negative *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Bacteroides fragilis*<sub>[DS3]</sub> at concentrations of 5.0-21.4 μM. More recently, Carballeira *et al.* determined that 2-HDA and its analog 2,6-hexadecadiynoic acid (2,6-HDA) were active

against *Mycobacterium tuberculosis* displaying minimum inhibitory concentrations (MICs) of 141-145  $\mu\text{M}$  (Carballeira et al., 2006). 2-Octadecynoic acid (2-ODA) was other acetylenic acid that was evaluated as an antimycobacterial agent (Morbidoni et al., 2006). According to that study, 2-ODA and its metabolites displayed the best antimycobacterial activity against *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG through the inhibition of fatty acid biosynthesis, such as fatty acid degradation and mycolic acid biosynthesis, which are fundamental pathways for the subsistence of mycobacteria (Morbidoni et al., 2006).

The antifungal properties of 2-HDA against several fungal strains, including *Aspergillus niger*, *Candida albicans*, and *Trichophyton mentagrophytes*, were also studied (Gershon and Shanks, 1978). The antifungal properties of 2-HDA has been attributed to its ability to inhibit the elongation of saturated and unsaturated fatty acid as well as its potential to inhibit the fatty acid acylation process, particularly triacylglycerol synthesis (Carballeira et al., 2006; Wood and Lee, 1981). The biological properties of 2-HDA provoked for Carballeira and collaborators to evaluate the antifungal properties of 2,6-HDA (Carballeira et al., 2006). They found that this fatty acid displayed increased activity against *C. albicans* [DS4] and *Cryptococcus neoformans* compared to the parent compounds 2-HDA and 6-HDA. Carballeira *et al.* postulated that both the inhibition of fungal fatty acid biosynthesis and inhibition of sphingolipid biosynthesis are responsible for the enhanced antifungal activity of 2,6-HDA (Carballeira et al., 2006).

In addition to its antibacterial and antifungal properties, 2-HDA has also shown antiprotozoal activity and inhibitory properties against protozoal enzymes. For example, Tasdemir *et al.* reported that 2-HDA effectively inhibited plasmodial FAS-II enzymes ( $\text{IC}_{50}$ 's between 1.5 and 13.9  $\mu\text{M}$ ) and arrests erythrocytic and liver stage plasmodium infections (Tasdemir et al., 2010). In addition, they showed that 2-HDA displays antiprotozoal activity against *Leishmania donovani* amastigotes ( $\text{IC}_{50} = 17.8 \mu\text{M}$ ), but no studies on key *L. donovani* enzymes amenable for therapeutic intervention were performed.

Aimed at studying the antiprotozoal properties of 2-HDA and other 2-AFAs, Carballeira and collaborators determined the antiprotozoal activity of a series of 2-AFAs, including 2-HDA (Carballeira et al., 2012). Results from this study revealed that 2-ODA and 2-HDA were the most potent antiprotozoal acids against *L. donovani* with  $\text{IC}_{50}$ 's of 11.0 and 17.8  $\mu\text{M}$ , respectively. Moreover, it was reported that the antiprotozoal activity of 2-HDA and 2-ODA was associated with their inhibitory properties against the *L. donovani* DNA topoisomerase IB enzyme (*LdTopIB*). 2-ODA and 2-HDA were inhibitory against *LdTopIB* at  $\text{IC}_{50}$  of 5.3 and 28.7  $\mu\text{M}$ , respectively (Carballeira et al., 2012). Although it has been suggested that the inhibition of *LdTopIB* could be a possible mechanism that explains the antileishmanial properties of the 2-alkynoic acids, Carballeira *et al.* did not discard the possibility that other mechanisms could be operative.

Despite the fact that the antimicrobial properties of 2-AFAs have been reported, further studies are needed to discover those structural characteristics that favor the antibacterial activity of 2-AFAs against multidrug-resistant bacteria. In this study, we synthesized four 2-AFAs by modifying the degree of unsaturation and carbon chain length. In addition, we prepared two alcohols and two tetrahydropyranyl ether analogues of 2-AFAs in order to

determine whether the carboxylic group in 2-AFAs is essential for the antibacterial activity of these compounds. The 2-AFAs presented here, were evaluated against both Gram-positive and Gram-negative bacteria including some methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Moreover, we investigated the relationship between antibacterial properties of 2-AFAs and their ability to form micelles. Finally, the cytotoxicity properties of 2-AFAs against normal peripheral blood mononuclear cells (PBMC) were also determined. Results from this study revealed that 2-AFAs were selective against bacteria and that there is no direct relationship between the antibacterial properties of these compounds and micelle formation. These results are important for future research in the area of the development of new antimicrobial agents that could be used to treat infections caused by multidrug-resistant bacteria in hospitals.

## 2. Materials and methods

### 2.1 Instrumentation

IR spectra were recorded on a Spectrum One Perkin Elmer FT-IR spectrophotometer.  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra were recorded on a Bruker DRX 500 spectrophotometer.  $^1\text{H}$ -NMR chemical shifts are reported relative to internal tetramethyl silane ( $\text{SiMe}_4$ ) and  $^{13}\text{C}$ -NMR chemical shifts are reported in parts per million (ppm) relative to  $\text{CDCl}_3$  (77.00 ppm). Mass spectra data was gathered by using a GC-MS (Agilent 5975C MS ChemStation; Agilent, Palo Alto, CA, USA) at 70 eV equipped with 30 m  $\times$  0.25 mm special performance capillary column (HP-5MS) of polymethylsiloxane cross-linked with 5% phenyl methylpolysiloxane. UV/Vis data were determined on a Beckman Coulter DU-530 spectrophotometer. Minimal inhibitory concentrations (MICs) were spectrophotometrically determined by using a Multiskan FC microplate reader (Fisher Scientific) at 620 nm.

### 2.2 Microorganisms

*Staphylococcus aureus* (ATCC 29213), *Staphylococcus saprophyticus* (ATCC 15305), *Bacillus cereus* (ATCC 10876), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), and Methicillin-resistant *S. aureus* (MRSA, ATCC 43300) were obtained from the American Type Culture Collection (Manassas, VA). Clinical isolates of MRSA (CIMRSA) were kindly donated by a community hospital in San Juan, Puerto Rico. Stock cultures were kept on blood agar (TSA with 5% sheep blood, Remel and Oxoid Microbiology Products, Lenexa, KS). Subcultures were incubated for 18-24 h on TSA at 37°C. Suspension cultures were prepared by inoculation of single colonies in 5 mL Trypticase Soy Broth (TSB, BD Diagnostic Systems, Franklin Lakes, NJ). Prior to preparation of susceptibility assays, bacteria cells were re-suspended in TSB and visually standardized by using 0.5 McFarland standard solution, which provided an equivalent concentration of  $1.0 \times 10^8$  CFU/mL.

### 2.3 Diagnostic tests for identification of *S. aureus*

To confirm the identity of *S. aureus*, MRSA (ATCC 43300) and two CIMRSA strains were tested for fermentation on mannitol salt agar (MSA, BD Diagnostic System, Franklin Lakes, NJ). A positive fermentation result was recorded as growth of yellow colonies on MSA surrounded by yellow zones after 24 h of incubation at 37°C.

The coagulase test was performed with plasma rabbit following the recommendations of the manufacturer (Becton, Dickinson and Company, Sparks, MD). Results were recorded after 4 and 24 hours of incubation at 37°C using *S. aureus* (ATCC 25923) as positive control and *S. saprophyticus* (ATCC 15305) as negative control. Small organized clots were recorded as a positive result.

## 2.4 Susceptibility testing

A modified version of the microdilution method outlined by the CLSI was used (CLSI, 2006). 2-AFAs were dissolved in 95% ethanol, serially diluted with sterile TSB, and transferred to a flat-bottomed microplate wells that were previously inoculated with 10 µL of TSB solution containing  $4.5 \times 10^5$  colony-forming units (CFU<sub>[DS5]</sub>). The wells were inspected spectrophotometrically (620 nm) using both a positive control well (containing the bacterial inoculated TSB but not the fatty acid solution) and a negative control well (containing only TSB) for comparison. The minimum inhibitory concentration (MIC) was considered to be the concentration at which 2-alkynoic acids prevented turbidity in the well after incubation for 18-24 h at 37°C.

## 2.5 Determination of bactericidal concentration (MBC)

Once the MIC experiments were performed, MBC was determined by removing approximately 10 µL of the TSB containing 2-AFA from four microplate wells whose turbidity was not observed (starting from the well where MIC was determined) and sub-culturing onto fresh TSA plate containing 5% sheep blood (Remel and Oxoid Microbiology Products, Lenexa, KS). TSA plates were incubated for a further 18-24 h at 37°C. Any growth observed from TSA plates was designated as an ineffective bactericidal concentration of 2-AFA<sub>[DS6]</sub>.

## 2.6 Cytotoxicity testing

Peripheral blood mononuclear cells (PBMC) were used for this assay. The cells were cultured in culture medium supplemented with interleukin-2 (IL-2). PBMC was seeded into a 96-well microplate ( $5 \times 10^4$  cells/ 200 µL/ well) and fatty acids were added to the cell cultures. The final concentrations of fatty acids ranged from 25 to 400 µg/mL. The cells were incubated at 37°C for 3 days in humidified 5% CO<sub>2</sub> incubator. The cytotoxicity of the cells was evaluated by MTT assay.

## 2.7 Synthesis of 2-HDA, 2-ODA , and 2-ICA

The synthesis of 2-HDA, 2-ODA, and 2-icosanoic acid (2-ICA) followed a previously published procedure (Carballeira et al., 2012; Tasdemir et al., 2010). In general, the above-mentioned AFAs were prepared by reacting the commercially available 1-pentadecyne, 1-heptadecyne, or 1-nonadecyne with *n*-BuLi in THF at -70°C, quenched with CO<sub>2</sub> and subsequently protonated with NH<sub>4</sub>Cl (Figure 1). The 2-HDA, 2-ODA, and 2-ICA were obtained in 40-79% yield. The spectral data of 2-HDA and 2-ODA were in agreement with those previously reported (Morbidoni et al., 2006; Tasdemir et al., 2010).

**2.7.1 Synthesis of 2-ICA**—2-ICA was obtained in a 40% yield as a white solid from the reaction of 0.550 g (2.08 mmol), *n*-BuLi (2.5 M, 3.75 mmol) in dry hexane (1.50 mL), and subsequent quenching with CO<sub>2</sub> according to the general procedure described above. The spectral data of 2-ICA is described below: M.p. 54–56°C. IR (KBr)  $\nu_{\max}$ : 3000–2840, 2963, 2916, 2844, 2255, 1671, 1463, 1432, 1317, 1281, 1083, 910, 796, 754, 713, 613 cm<sup>-1</sup>; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  2.35 (2H, t), 1.58 (2H, m), 1.45–1.35 (2H, m) 1.35–1.18 (26H, m), 0.88 (3H, t); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  157.96 (s, C-1), 92.53 (s, C-3), 72.61 (s, C-2), 31.31 (t), 29.68 (t) 29.65 (t), 29.62 (t), 29.56 (t), 29.39 (t), 29.35 (t), 28.99 (t), 28.81 (t), 23.37 (t), 22.68 (t), 18.74 (t), 14.11 (q, C-20). GC-MS (70ev) *m/z* (relative intensity) for the methyl ester derivative; 291(M<sup>+</sup>,12), 195(4), 181(4), 154(76), 151(6), 150(9), 149(9), 141(7), 140(35), 139(17), 136(13), 135(20), 126(7), 125(13), 123(12), 122(23), 121(31), 114(20), 113(13), 111(28), 109(24), 108(29), 107(34), 100(63), 97(19), 96(15), 95(44), 94(45), 93(47), 91(19), 87(7), 85(10), 83(22), 82(19), 81(58), 80(34), 79(81), 77(19), 71(19), 69(45), 68(22), 67(54), 66(23), 65(10), 57(71), 56(20), 55(100); HRMS (APCI) Calcd for C<sub>12</sub>H<sub>21</sub>O<sub>2</sub> [M+H]<sup>+</sup> 309.27881, found 309.27835.

## 2.8 Synthesis of 2,6-HDA and 2,9-HDA

2,6-HDA and 2,9-HDA were prepared following a previously published procedure (Carballeira et al., 2006) The above-mentioned diunsaturated AFAs were prepared by reacting the lithium acetylide of 1,5-hexadiyne with 1-bromononane and the lithium acetylide of 1,8-nonadiyne with 1-bromohexane to obtain both 1,5-pentadecadiyne and 1,8-pentadecadiyne (Figure 2). Coupling of the lithium acetylides of 1,5-pentadecadiyne and 1,8-pentadecadiyne with CO<sub>2</sub> at -70°C and subsequent protonation with NH<sub>4</sub>Cl afforded the desired 2,6-HDA and 2,9-HDA. The spectral data of 2,6-HDA and 2,9-HDA were in agreement with those previously reported (Carballeira et al., 2006).

## 2.9 Synthesis of 2-(2-alkynyloxy)-tetrahydro-2H-pyran compounds

To a stirred solution of tetrahydro-2-(2-propionyloxy)-2H-pyran (7.13 mmol) in dry THF (20 mL), *n*-BuLi (2.5 M, 17.83 mmol) in dry hexane (7.10 mL) was added dropwise while keeping the temperature at -78°C. After 30 min, HMPA (5.0 mL) and 1-bromotridecane (7.13 mmol) or 1-bromopentadecane (7.13 mmol) was added dropwise to the reaction mixture while maintaining the temperature at 78°C. After 24 h, the reaction mixture was worked up by pouring a large volume of water and extracting with diethyl ether (2 × 20 mL). The organic layer was washed with brine (1 × 20 mL) before drying (MgSO<sub>4</sub>). Filtration, rotoevaporation of the solvent and column purification with hexane-diethyl ether (9:1 v/v) solution afforded 1.38 g (60% yield) of 2-(2-hexadecyloxy)-tetrahydro-2H-pyran and 1.89 g (76% yield) of 2-(2-octadecyloxy)-tetrahydro-2H-pyran.

### 2.9.1 2-(2-Hexadecyloxy)-tetrahydro-2H-pyran (2-HDOTHP)

2-(2-Hexadecyloxy)-tetrahydro-2H-pyran was obtained in a 60% yield as colorless oil from the reaction of 1.00 mL of tetrahydro-2-(2-propynyloxy)-2H-pyran (1.00 g, 7.13 mmol) and 1.82 mL of 1-bromotridecane (7.13 mmol) according to the general procedure described above. IR (neat)  $\nu_{\max}$ : 2926, 2854, 2225, 1466, 1345, 1202, 1118, 1133, 1079, 1054, 1025 cm<sup>-1</sup>; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  4.81 (1H, t, J = 3.3 Hz), 4.24 (2H, m), 3.84 (1H, m); 3.52 (1H, m), 2.20 (2H, m); 1.89–1.25 (33H, m), 0.87 (3H, t, J = 6.4 Hz); <sup>13</sup>C NMR (75



(MHz)  $\delta$  96.58, 86.78 (s, C-3), 75.64 (s, C-2), 61.96, 54.62 (t, C-1), 31.90, 30.27, 29.64 (x 3), 29.61, 29.52, 29.34, 29.12, 28.87, 28.58, 25.36, 22.67, 19.11, 18.80, 14.10 (q, C-16). GC-MS (70ev)  $m/z$  (relative intensity); 322 (M<sup>+</sup>, 0.11), 279 (0.13), 209 (0.55), 149 (2), 135 (4), 121 (7), 111 (17), 95 (46), 85 (100), 81 (49), 77 (11), 67 (57), 55 (90).

### 2.9.2 2-(2-Octadecyloxy)-tetrahydro-2H-pyran (2-ODOTHP)—2-(2-

Octadecyloxy)-tetrahydro-2H-pyran was obtained in a 76% yield as colorless oil from the reaction 1.00 mL of tetrahydro-2-(2-propynoxy)-2H-pyran (1.00 g, 7.13 mmol) and 2.00 mL of 1-bromopentadecane (7.13 mmol) according to the general procedure described above. IR (neat)  $\nu_{\max}$ : 2925, 2854, 2222, 1466, 1345, 1201, 1132, 1118, 1079, 1053, 1025  $\text{cm}^{-1}$ ; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  4.81 (1H, t, J = 3.27 Hz), 4.24 (2H, m), 3.84 (1H, m), 3.52 (1H, m), 2.20 (2H, m); 1.89-1.25 (32H, m), 0.87 (3H, t, J = 6.69 Hz); <sup>13</sup>C NMR (75 MHz)  $\delta$  96.57, 86.77 (s, C-3), 75.64 (s, C-2), 61.95, 54.62 (t, C-1), 31.90, 30.27, 29.67 (x 3), 29.61, 29.52, 29.348, 29.12, 28.87, 28.75, 28.59, 28.16, 25.36, 22.67, 19.10, 18.80, 14.10 (q, C-18). GC-MS (70ev)  $m/z$  (relative intensity); 350 (M<sup>+</sup>, 0.55), 295 (0.68), 279 (1), 209 (2), 149 (5), 135 (14), 121 (17), 111 (39), 95 (59), 85 (100), 81 (55), 77 (9), 67 (47), 55 (81).

## 2.10 Synthesis 2-HDOH and 2-ODOH

2-(2-alkynyloxy)-tetrahydro-2H-pyran compounds (4.29-5.08 mmol) in methanol (35.0-40.0 mL), and catalytic amounts of p-toluenesulfonic acid (PTSA) were stirred at 45°C for 24 h. The excess PTSA was removed through a liquid-liquid extraction using a saturated solution of NaHCO<sub>3</sub> and diethyl ether (2 × 30 mL). Drying of the organic phase (MgSO<sub>4</sub>) and subsequent rotoevaporation of the solvent gave 2-alkynols as white solids.

**2.10.1 2-Hexadecyn-1-ol (2-HDOH)**—2-HDOH was obtained as white solid in 67% yield from the reaction of 1.38 g (4.29 mmol) of 2-(2-hexadecyloxy)-tetrahydro-2H-pyran and catalytic amounts of PTSA using the procedure described above. M.p. 40-43°C, IR (neat)  $\nu_{\max}$ : 3314, 2925, 2854, 2218, 1466, 1378, 1022  $\text{cm}^{-1}$ ; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  4.24 (2H, s), 2.20 (2H, tt, J = 7.05, J = 2.11), 1.72 (1H, s), 1.59-1.21 (22H, m), 0.87 (3H, t, J = 6.63 Hz); <sup>13</sup>C NMR (75 MHz)  $\delta$  86.64 (s), 78.21 (s), 51.38 (t, C-1), 31.90, 29.66, 29.64 (x 3), 29.50, 29.34, 29.13, 28.86, 28.58, 22.67, 18.70, 14.11 (q, C-16). GC-MS (70ev)  $m/z$  (relative intensity); 238 (M<sup>+</sup>, 0.01), 223 (M<sup>+</sup>-15, 0.02), 207 (1), 191 (0.6), 177 (0.4), 149 (3), 135 (9), 121 (14), 111 (19), 107 (13), 93 (40), 81 (56), 67 (65), 55 (100). HRMS (APCI) Calcd for C<sub>16</sub>H<sub>31</sub>O [M+H]<sup>+</sup> 239.23694, found 239.23661.

**2.10.2 2-Octadecyn-1-ol (2-ODOH)**—2-ODOH was obtained as white solid in 65% yield from the reaction of 1.78 g (5.08 mmol) and catalytic amounts of PTSA using the procedure described above. M.p. 45-47°C.  $\nu_{\max}$ : 3184, 2917, 2850, 2219, 1471, 1367, 1022  $\text{cm}^{-1}$ ; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  4.24 (2H, s), 2.20 (2H, tt, J = 7.06, J = 2.16 Hz), 1.82 (1H, s), 1.72-1.24 (26H, m), 0.87 (3H, t, J = 6.70 Hz); <sup>13</sup>C NMR (75 MHz)  $\delta$  86.62 (s), 78.22 (s), 51.38 (C-1, t), 31.90, 29.67, 29.64 (x 3), 29.61, 29.51, 29.42, 29.34, 29.13, 28.86, 28.58, 22.67, 19.28, 14.11 (C-18, q). GC-MS (70ev)  $m/z$  (relative intensity); 266 (M<sup>+</sup>, 0.31); 251 (M<sup>+</sup>-15, 0.25), 235 (4), 219 (2), 205 (1), 149 (8), 135 (22), 121 (30), 111 (43), 107 (22),

93 (53), 81 (75), 67 (73), 55 (100); HRMS (APCI) Calcd for C<sub>18</sub>H<sub>35</sub>O [M+H]<sup>+</sup> 267.26824, found 267.26791.

### 2.11 Determination of the critical micelle concentration (CMC)

The CMC of the fatty acids was assessed as previously described by Courtney et al (Courtney et al., 1986). In general, the fatty acids were dissolved in 95% ethanol<sub>[DS7]</sub> and serially diluted (0.1-1000 µg/mL) in 1X phosphate-buffered saline (1X PBS) containing rhodamine 6G at  $2.5 \times 10^{-6}$  M. The wavelength of maximum absorption was determined for each dilution using a Beckman Coulter DU-530 spectrophotometer and plotted as a function of the fatty acid concentration. The CMC value was described as the point at which the wavelength of maximum absorption first deviated from linearity.

### 2.12 Statistical analyses

All dose-response curves were carried out by using GraphPad Prism<sup>®</sup> (v 5.0) biostatistics software (San Diego, CA) to determine IC<sub>50</sub>. The IC<sub>50</sub> was defined as the concentration of the 2-alkynoic acid that inhibits 50% of the bacterial growth.

## 3. Results and discussion

In a prior study, we synthesized novel 2-AFAs in order to study their antifungal properties (Carballeira et al., 2006). In that report, we determined that the addition of a triple bond at C-6 in 2-HDA increased its fungitoxicity, while the addition of a triple bond at C-9 was not effective in increasing its antifungal activity. By using a similar approach as described before (Carballeira et al., 2012; Carballeira et al., 2006; Tasdemir et al., 2010), we synthesized 2-AFAs and other synthetic analogues in order to determine those structural characteristics that favor the antibacterial activity of 2-AFAs. In this study, we prepared 2-HDA, 2-ODA, 2-ICA, 2,6-HDA, and 2,9-HDA according to Schemes 1 and 2. The syntheses of 2-HDA, 2-ODA, and 2-ICA were successfully completed in one step and in 40-79% yields (Scheme 1). The synthesis of these 2-AFAs was accomplished by reacting commercially available 1-pentadecyne, 1-heptadecyne or 1-nonadecyne with *n*-BuLi in THF followed by quenching with CO<sub>2</sub> and final protonation with NH<sub>4</sub>Cl.

In the case of 2,6-HDA and 2,9-HDA, these AFAs were conveniently synthesized by using either 1,5-hexadiyne (50% in pentane) or 1,8-nonadiyne as starting materials (Scheme 2). The 2,6-HDA was prepared in two steps with an overall yield of 18%. The synthesis started with the coupling of 1,5-hexadiyne with 1-bromononane using *n*-BuLi in THF/HMPA at -78°C, subsequent coupling of the lithium acetylide of 1,5-pentadecadiyne with CO<sub>2</sub>, and final protonation with NH<sub>4</sub>Cl affording the desired dimethylene-interrupted 2,6-HDA. 2,9-HDA was prepared with an overall yield of 11% following a similar synthetic strategy described above, with the exception that this synthesis started with 1,8-nonadecyne. This diyne was coupled with 1-bromohexane using *n*-BuLi in THF/HMPA at -78°C, subsequently reacted with *n*-BuLi and CO<sub>2</sub>, and finally protonated with NH<sub>4</sub>Cl to afford the desired 2,9-HDA.

Other analogues of 2-AFAs including tetrahydropyranyl ethers and 2-alkynols were also prepared to establish a SAR aimed at determining the compound with better cytotoxicity



towards bacteria. In this study, we prepared the tetrahydropyranyl ethers 2-HDOTHP and 2-ODOTHP, which served as starting materials for the preparation of the 2-alkynols 2-HDOH and 2-ODOH as depicted in Scheme 3. For the preparation of 2-HDOTHP and 2-ODOTHP, the 2-(2-propynyloxy)-tetrahydro-2H-pyran was coupled with either 1-bromotridecane or 1-bromopentadecane to produce 2-HDOTHP and 2-ODOTHP in 60 and 76% yields, respectively. The removal of the tetrahydropyranyl protecting group in both 2-HDOTHP and 2-ODOTHP was successfully accomplished with PTSA in methanol at 45°C, producing the desired 2-alkynols 2-HDOH and 2-ODOH in 67 and 65% yields, respectively.

The antibacterial activity of the 2-AFAs and their analogues against *S. aureus*, *S. saprophyticus*, *B. cereus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* were determined by using a modified version of the microdilution method outlined by the CLSI (CLSI, 2006). The Kirby-Bauer method (CLSI, 2012) was also used for determining the antibacterial activity of 2-AFAs against several *S. aureus* strains (data not shown<sub>[DS8]</sub>). Altogether these bacteria were selected for this study because they are associated to nosocomial infections such as skin infections, urinary tract infections, food poisoning, respiratory tract infections, and cross-infections when using contaminated medical equipment. Table 1 shows the MICs and IC<sub>50</sub>s of 2-AFAs and their analogues against the above mentioned bacteria. Methicillin and ciprofloxacin were used as positive controls. Among the compounds tested, 2-HDA exhibited the lowest<sub>[DS9]</sub> MIC/IC<sub>50</sub> values against Gram-positive *S. aureus*, *S. saprophyticus*, and *B. cereus* and against the Gram-negative *K. pneumoniae* <sub>[DS10]</sub> and *P. aeruginosa* with MICs between 7.8 and 125 µg/mL. 2-ODA also displayed good antibacterial activity against the bacteria mentioned above. It can be noted from the MICs/IC<sub>50</sub> values in Table 1 that 2-HDA showed a broader antibacterial activity when compared to the other analogues. Also, it was noted that the increase in the carbon chain length in 2-AFA, decreases its antibacterial activity<sub>[DS11]</sub>. This is the case of 2-ICA, a 20-carbon chain length AFA, which did not show antibacterial activity against any bacteria presented in Table 1.

Unlike our previous antifungal work, the addition of a second triple bond at C-6 in 2-HDA, decreases its antibacterial activity. Moreover, it can be noted from Table 1 that the addition of a second triple bond at C-9 decreases more notably the antibacterial activity of 2-HDA than the addition of a second triple bond at C-6. According to Morbidoni et al., 2-HDA is specific against bacteria because the acid inhibits the activity of InhA, the enoyl-ACP reductase of the type II fatty acid synthase (FASII) that catalyzes the NADH-specific reduction of 2-trans-enoyl-ACP (Morbidoni et al., 2006). Based on this finding, we believe that the addition of a second triple bond at either C-6 or C-9 in 2-HDA do not favor the interactions between either 2,6-HDA or 2,9-HDA, and the InhA enzyme.

The data in Table 1 also demonstrate that the presence of a carboxylic acid moiety in 2-AFAs is important for their antibacterial activity. For example, 2-HDA displayed good biological activity against several Gram-positive and Gram-negative bacteria, but the corresponding alcohol analog, 2-HDOH, was not active against any of the bacterial strains presented in Table 1. A similar pattern was observed with 2-ODA and 2-ODOH. Tetrahydropyranyl ethers, such as 2-HDOTHP and 2-ODOTHP, were also inactive against all tested bacteria (Table 1), which suggests that the presence of an ether functional group

does not favor the antibacterial activity of 2-AFA analogues. In general, as antibacterial agents, the 2-AFAs followed the order: 2-HDA > 2-ODA > 2,6-HDA > 2,9-HDA > 2-ICA. Altogether these findings support that the carboxylic acid moiety as well as the triple bond at C-2 are needed for the antibacterial activity of 2-AFAs.

MRSA were included in our study, because the reduction of infections caused by this bacteria in hospital settings represents a priority for the Center for Control Diseases and Prevention (CDC), since this threat is responsible for the largest outbreak of hospital acquired infections (CDC, 2013; Klevens et al., 2007). As part of this study, we determined the antibacterial activity of 2-AFAs against three pathogenic MRSA strains: one commercial strain and two bacterial strains isolated from clinical patients. All three MRSA strains fermented mannitol and were coagulase positive, which confirmed them as *S. aureus*. Moreover, we confirmed the resistance pattern of the clinical isolates of MRSA (CIMRSA) to both methicillin and ciprofloxacin by using the Kirby-Bauer disk diffusion method (CLSI, 2012), where *S. aureus* ATCC 29213 was used as the standard (data not shown). Our disk diffusion results demonstrate that the two CIMRSA strains tested were both methicillin-resistant and ciprofloxacin-resistant, which supports that these bacteria strains are multidrug resistant organisms (MRO). Moreover, our disk diffusion results showed that all MRSA strains tested have different resistance pattern to both methicillin and ciprofloxacin, which suggest that these bacteria are not the same. Table 2, shows the antibacterial activity of several 2-AFAs against MRSA ATCC 43300 and the two CIMRSA strains. It can be observed from Table 2 that both 2-HDA and 2-ODA are more active against all MRSA strains tested. Moreover, these compounds demonstrated to be comparable or more effective as antibacterial compounds than either methicillin or ciprofloxacin (Table 2). Results in Table 2 also show that the addition of a second triple bond at either C-6 or C-9 do not favor the antibacterial activity of 2-AFAs, which is consistent with the results displayed in Table 1. The bactericidal effect of 2-AFAs against CIMRSA strains were also evaluated. Table 3 shows that 2-HDA and 2-ODA were bactericidal against CIMRSA 1 and 2 at concentrations of 7.8 and 15.6 µg/mL, respectively. In the case of 2,6-HDA and 2,9-HDA, these compounds displayed bactericidal effect against CIMRSA strains at concentrations of 125-500 µg/mL.

Cytotoxicity of 2-AFAs was determined against PBMC isolated from healthy volunteers. As shown in Table 2, 2-HDA was the most toxic of the four 2-AFAs towards PBMC. However, 2-HDA showed the highest selectivity towards CIMRSA strains, which make it a more therapeutically valuable compound. This result is in agreement with findings published by Carballeira et al., where they reported that 2-HDA showed the highest therapeutic index (TI) as antiprotozoal agent (Carballeira et al., 2012). The marked difference between the cytotoxicity of 2-AFAs towards PBMC and their antibacterial activities can be due to the fact that the FASI complex in microsomal systems is not inhibited by 2-AFAs (Morbidoni et al., 2006).

Since several fatty acids tend to form micellar aggregates in aqueous media (Courtney et al., 1986; Orellano et al., 2013), we determined the CMC of several 2-AFAs in order to investigate whether micellar formation is associated with their antibacterial activity against MRSA. To the best of our knowledge, the CMCs of 2-AFAs have not been reported before.

Several studies have demonstrated a relationship between the CMC of amphiphilic compounds and their biological activity (Courtney et al., 1986; Smith et al., 1979). Recently, Carballeira and collaborators determined that the anticancer activities of  $\alpha$ -methoxylated fatty acids are related to their CMCs (Orellano et al., 2013). In contrast to other studies, the results in Table 4 showed that the antibacterial activity of 2-HDA and 2-ODA is not mediated by micelle formation, suggesting that the free monomeric form of these 2-AFAs are responsible for their biological activity against MRSA. These results strongly support the findings reported by Wicken and collaborators (Wicken et al., 1986). In this report they demonstrated that the free monomer of lipoteichoic acids (LTAs), amphiphiles isolated from Gram-positive organisms, represents the major configuration of extracellular LTAs in bacterial culture fluids (Wicken et al., 1986). It can be noted from Table 4 that there is a relationship between the decline in the antibacterial activity of 2,6-HDA and its CMC. Wicken et al. concluded that it is important for micellar aggregates to be separated in their respective monomer units to allow the intercalation of the monomer into membranes (Wicken et al., 1986). Therefore, we hypothesize that the decline in the concentration of the monomer units of 2,6-HDA in aqueous medium, do not favor the hydrophobic interactions between this 2-AFA and the bacterial cell wall. These membrane-fatty acid interactions would presumably be facilitated at high concentrations of the free fatty acid. On the other hand, it can be observed that the CMC of 2,9-HDA is ten-fold higher than the CMC of 2,6-HDA suggesting that the presence of a triple bond at C-9 in 2-HDA does not favor the micelle formation. Therefore, the antibacterial activity of 2,9-HDA can be mediated by a non-micelle-dependent mechanism.

Further experiments are required to understand the mechanism of action of antibacterial 2-AFAs. Although the InhA enzyme has been proposed as a possible molecular target (Morbidoni et al., 2006), other possible mechanisms of action for the 2-AFAs have been explored to explain their antibacterial properties. For example, Konthikamee et al. demonstrated that the absorption and utilization of 2-AFAs in the synthesis of bacterial phospholipids is a possible mechanism of action of these compounds (Konthikamee et al., 1982). These authors explained that the mechanism above takes place as a result of the activation of 2-AFAs to the acyl coenzyme A thioester, which act as substrate for the acyl coenzyme A phospholipid acyl transferase of bacterial cells (Konthikamee et al., 1982).

In conclusion, we performed three synthetic strategies aimed at determining those structural characteristics that are needed to prepare 2-AFAs with better antibacterial activity. Our results demonstrated that the presence of a triple bond at C-2 as well as the carboxylic acid moiety are important functionalities for the antibacterial activity of 2-AFAs against Gram-positive and Gram-negative bacteria, including clinical isolates of methicillin-resistant *S. aureus*. Moreover, we determined that by increasing the carbon chain length in 2-AFAs, their antibacterial activity decreases. Our results also demonstrated that the antibacterial activity of 2-AFAs is not mediated by micellar aggregates formation. This study will surely impact our understanding regarding the antibacterial properties of 2-AFAs, and highlight 2-HDA as a novel agent against nosocomial bacterial infections.

## Acknowledgments

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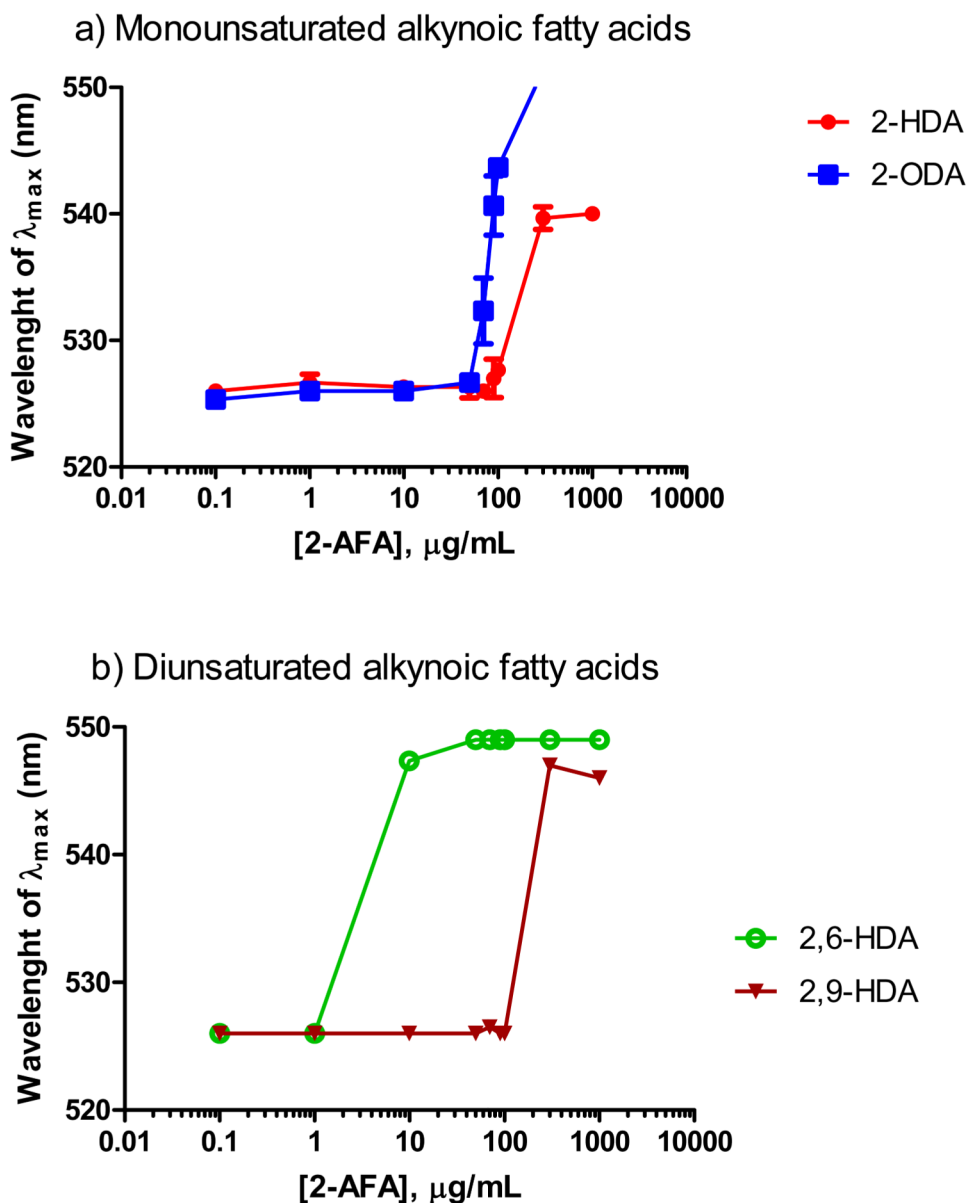
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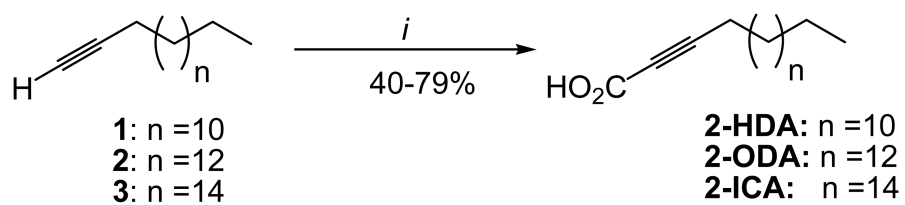
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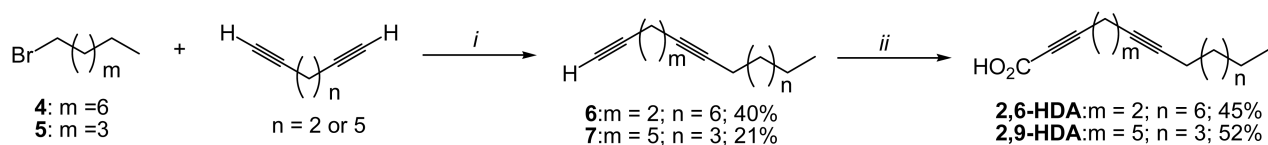




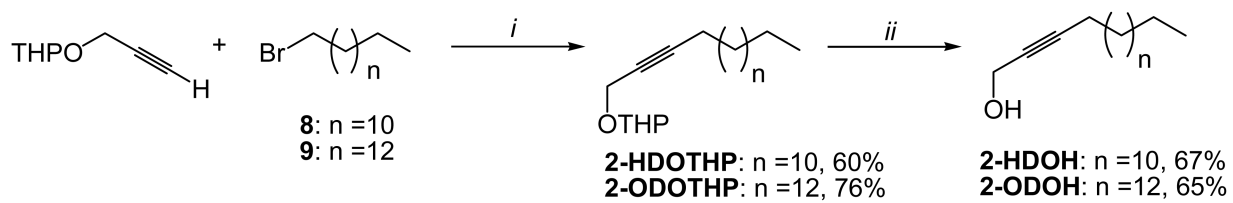
**Figure 1.** Wavelength of maximum absorption of rhodamine 6G with increasing concentrations of 2-AFAs in 1X PBS at 25°C. a) Monounsaturated alkynoic fatty acids; b) Diunsaturated alkynoic fatty acids. Values are reported as means  $\pm$  SEM ( $N = 3_{\text{[DS12]}}$ ).

**Scheme 1.**

Total synthesis of 2-HDA, 2-ODA, and 2-ICA. *i*) *n*-BuLi, THF,  $-78^{\circ}\text{C}$ , carbon dioxide( $\text{CO}_2$ ),  $\text{NH}_4\text{Cl}$ .

**Scheme 2.**

Total synthesis of 2,6-HDA and 2,9-HDA. *i*) *n*-BuLi, THF,  $-78^{\circ}\text{C}$ , HMPA, 24 h; *ii*) *n*-BuLi, THF,  $-78^{\circ}\text{C}$ , carbon dioxide ( $\text{CO}_2$ ),  $\text{NH}_4\text{Cl}$ .

**Scheme 3.**

Total synthesis of 2-HDOH and 2-ODOH. *i)*  $n$ -BuLi, THF, -78°C, HMPA, 24 h; *ii)* PTSA, MeOH, 45°C, 24h.

**Table 1**  
**Antibacterial activity of 2-AFAs and other analogues against Gram-positive and Gram-negative bacteria at 37°C after 18-24 h<sup>a</sup>**

Compound	MIC/IC <sub>50</sub> ± SEM (µg/mL)							
	<i>S. aureus</i> (ATCC 29213)	<i>S. saprophyticus</i> (ATCC 15305)	<i>B. cereus</i> (ATCC 10876)	<i>E. coli</i> (ATCC 25922)	<i>K. pneumoniae</i> (ATCC 13883)	<i>P. aeruginosa</i> (ATCC 27853)		
<b>2-HDA</b>	15.6/6.2 ± 0.9	15.6/8.8 ± 0.1	31.3/17.2 ± 1.0	>1,000	7.8/6.1 ± 0.1	125/87.8 ± 1.1		
<b>2-ODA</b>	15.6/8.1 ± 1.0	125/68.8 ± 3.5	15.6/11.9 ± 0.8	>1,000	7.8/5.6 ± 0.2	>1,000		
<b>2-ICA</b>	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000		
<b>2,6-HDA</b>	31.3/14.1 ± 0.1	62.5/21.5 ± 0.6	62.5/46.1 ± 0.3	>1,000	62.5/31.2 ± 0.5	250/132.3 ± 3.5		
<b>2,9-HDA</b>	250/94.0 ± 0.9	125/61.5 ± 1.2	62.5/34.2 ± 0.3	>1,000	250/135.4 ± 0.7	>1,000		
<b>2-HDOTHP</b>	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000		
<b>2-ODOTHP</b>	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000		
<b>2-HDOH</b>	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000		
<b>2-ODOH</b>	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000		
<b>METH</b>	0.06/0.03 ± 0.01	0.12/0.06 ± 0.01	62.5/38.2 ± 0.7	>1,000	0.98/0.36 ± 0.01	>1,000		
<b>CIPRO</b>	0.25/0.14 ± 0.01	<0.002	0.05/0.03 ± 0.01	<0.008	0.24/0.13 ± 0.01	0.24/0.11 ± 0.01		

<sup>a</sup>Experiments were performed in triplicate (N=3). Meth, Methicillin, Cipro, Ciprofloxacin.

**Table 2**  
**Antibacterial activity of several 2-AFAs against MRSA ATCC 43300 and CIMRSA strains.** <sup>a</sup>

Compound	MIC/IC <sub>50</sub> ± SEM (µg/mL)/Therapeutic Index (TI) <sup>b</sup>			PBMC cytotoxicity, IC <sub>50</sub> ± SEM (µg/mL)
	MRSA ATCC 43300	CIMRSA 1 <sup>c</sup>	CIMRSA 2 <sup>c</sup>	
<b>2-HDA</b>	15.6/9.4 ± 0.1/6.1	3.9/3.3 ± 0.1/17.3	3.9/3.4 ± 1.0/16.8	57.2 ± 0.1
<b>2-ODA</b>	7.8/5.5 ± 1.0/10.7	7.8/4.3 ± 1.0/13.7	7.8 /5.5 ± 1.0/10.7	58.9 ± 0.1
<b>2,6-HDA</b>	62.5/22.3 ± 0.3/2.8	62.5/39.5 ± 0.9/1.6	62.5/22.4 ± 0.4/2.6	63.4 ± 0.3
<b>2,9-HDA</b>	500/203.5 ± 2.6/0.4	250/102.1 ± 2.4/0.9	250/ 76.0 ± 2.4/1.2	93.3 ± 0.4
<b>Methicillin</b>	1.95/0.98 ± 0.02/87.9	7.8/2.2 ± 0.7/39.1	31.3/11.8 ± 3.9/7.3	86.1 ± 0.5
<b>Ciprofloxacin</b>	0.50/0.20 ± 0.09/332	15.6/10.4 ± 3.7/6.4	3.9/2.9 ± 1.2/22.9	66.4 ± 0.6

<sup>a</sup>Experiments were performed in triplicate (N =3). Methicillin and ciprofloxacin were used as positive controls.

<sup>b</sup>TI was determined by dividing PBMC cytotoxicity by the inhibitory concentration (IC<sub>50</sub>) of the drug.

<sup>c</sup>CIMRSA = Clinical isolate of methicillin-resistant *S. aureus*.



**Table 3**Bactericidal activity of 2-AFAs against CIMRSA strains.<sup>a</sup>

Compound	MBC, µg/mL	
	CIMRSA 1 <sup>b</sup>	CIMRSA 2 <sup>b</sup>
2-HDA	7.8	7.8
2-ODA	15.6	15.6
2,6-HDA	125	250
2,9-HDA	500	500

<sup>a</sup>Experiments were carried out in triplicate (N = 3).<sup>b</sup>CIMRSA = Clinical isolate of methicillin-resistant *S. aureus*.

**Table 4**Relationship between the antibacterial activity of several 2-AFAs against MRSA and their CMC. <sup>a,b</sup>

Compound	IC <sub>50</sub> ± SEM (µg/mL)			CMC (µg/mL)
	MRSA ATCC 43300	CIMRSA 1 <sup>c</sup>	CIMRSA 2 <sup>c</sup>	
<b>2-HDA</b>	9.4 ± 0.1	3.3 ± 0.1	3.4 ± 1.0	> 90
<b>2-ODA</b>	5.5 ± 1.0	4.3 ± 1.0	5.5 ± 1.0	50-70
<b>2,6-HDA</b>	22.3 ± 0.3	39.5 ± 0.9	22.4 ± 0.4	1-10
<b>2,9-HDA</b>	203.5 ± 2.6	102.1 ± 2.4	76.0 ± 2.4	> 100

<sup>a</sup>The CMC was determined as described in materials and methods.<sup>b</sup>Experiments were performed in triplicate (N = 3).<sup>c</sup>Clinical isolates of methicillin-resistant *S. aureus* (MRSA).