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Paula Bourke
Technological University Dublin, paula.bourke@tudublin.ie

Patricia Nobmann
Technological University Dublin, Patricia.Nobmann@tudublin.ie

Julie Dunne
Technological University Dublin, Julie.Dunne@tudublin.ie

Gary Henehan
Technological University Dublin, gary.henehan@tudublin.ie

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***In vitro* antimicrobial activity and mechanism of action of novel carbohydrate fatty acid derivatives against *Staphylococcus aureus* and MRSA**

Patricia Nobmann, Paula Bourke*, Julie Dunne and Gary Henehan

School of Food Science and Environmental Health, Dublin Institute of Technology, Cathal Brugha Street, Dublin 1, Ireland

* Corresponding author. Tel: +353-1-4027594; Fax: +353-1-4024495; E-mail: paula.bourke@dit.ie

Running Title: Mechanism of Action of Novel Carbohydrate Fatty Acid Derivatives

ABSTRACT

Aims: This study investigates the antimicrobial activity and mode of action of novel carbohydrate fatty acid (CFA) derivatives against *Staphylococcus aureus* and methicillin-resistant *Staph. aureus* (MRSA).

Methods and Results: Minimum inhibitory concentrations (MIC) and the effect of CFA derivatives on lag phase were determined using a broth microdilution method. Lauric acid carbohydrate esters and corresponding ether analogs showed the greatest antimicrobial activity with MIC values between 0.04 to 0.16mmol l^{-1}. Leakage studies at 260nm following exposure to CFA derivatives at 4X MIC showed a significant increase in membrane permeability for all compounds, after *ca.* 15 minutes exposure except for the lauric beta ether CFA derivative. Further assessment using both BacLight and luminescence ATP assays, confirmed that an increase in membrane permeability and reduced metabolic activity was associated with CFA treatment.

Conclusions: All strains were significantly inhibited by the novel compounds studied and efficacy was related to specific structural features. Cell-membrane permeabilization was associated with CFA treatment and may account for at least a component of the mode of action of these compounds.

Significance and Impact of Study: This study reports the antimicrobial action of CFA compounds against a range of *Staph. aureus* and MRSA strains, and provides insights into their mode of action.

Keywords: *S. aureus;* MRSA; Carbohydrate fatty acid derivatives; Monolaurin; Antimicrobial activity; Mode of action; Membrane damage

1. INTRODUCTION

Staphylococcus aureus is an important human pathogen that can grow in inappropriately stored food causing a wide variety of diseases in humans either through toxin production or invasion. *Staph. aureus* may occur as a commensal on human skin; it can also be present in the anterior nares, with a mean carriage rate of 37.2% in the general population (Kluytmans *et al.*, 1997). *Staph. aureus* is also one of the most common causes of bloodstream infection (or bacteremia) (Petti and Fowler, 2003).

MRSA and antibiotic resistance is a global concern. *Staph. aureus* becomes methicillin resistant by the acquisition of the *mecA* gene which encodes a penicillin binding protein (PBP2a) with a low affinity for β-lactams, usually carried on a larger piece of DNA called a staphylococcal cassette chromosome *SCCmec* (Hartman and Tomasz, 1986). Infections caused by methicillin-resistant *Staph. aureus* (MRSA) are increasing in both hospital and community settings (Chopra, 2003). In Ireland there were 1,394 reports of *Staph. aureus* bacteraemia in 2007, of which 537 (38.5%) were MRSA (HPSC, 2007). Consequently, there has been considerable interest in discovering and developing new antistaphylococcal agents for potential therapeutic application (Chopra, 2003).

Carbohydrate fatty acid esters are biodegradable, non-toxic compounds currently being used as non-ionic surfactants in the food and health care industries. The antibacterial activity of fatty acid carbohydrate derivatives is increasingly of interest. Many authors have shown that these compounds are active against a range of pathogens (Monk *et al.*, 1996; Yang *et al.*, 2003; Devulapalle *et al.*, 2004; Ferrer *et al.*, 2005; Habulin *et al.*, 2008).

Unlike antibiotics, fatty acids and their derivatives have diverse modes of action that appear to be non-specific and development of resistance to these compounds has not been reported (Kabara and Marshall, 2005). Kitahara *et al.*, (2006) proposed lauric acid as a potential

antimicrobial material, suitable for external application, which could be combined with other antimicrobial agents. For example, Ved *et al.* (1990) demonstrated that monolaurin (ML), the glycerol monoester of lauric acid, acted synergistically with penicillin G at concentrations below its minimum micelle concentration. Kitahara *et al*., 2006, found that a lauric acid and gentamicin combination showed synergistic activity against MRSA.

The exact mode of action of CFAs on bacteria is unknown. However, it has been proposed, and is widely believed, that the cell membrane is the principal site of action of fatty acids and their esters (Kabara, 1993). Wang and Johnson (1992) found that monolaurin, a compound closely related to CFA esters, produced marked morphological changes in *L. monocytogenes* cells. While Bergsson *et al*. (2001) demonstrated that *Staph. aureus* was killed by fatty acids and derivatives, especially monocaprin, through disintegration of the cell membrane. The mechanism of the induction of autolysis by these types of compounds, remains to be elucidated, although it is suggested that they may disorganize the membrane structure and trigger an, as of yet unidentified, autolysin control system, possibly located in and/or on the cell membrane (Tsuchido, *et al*., 1993).

It has also been suggested that ML may affect the respiratory activity of cells by the inhibition of enzymes involved in oxygen uptake and/or inhibit the transport of amino acids into cells (Galbraith and Miller, 1973). Other researchers have reported that monolaurin at sub-MIC concentrations inhibited production of β-lactamases, toxic shock syndrome toxin 1 and other exoproteins in *Staph. aureus* at the level of transcription by interfering with signal transduction (Schlievert *et al*.,1992; Projan *et al*., 1994). Interference with signal transduction has been shown in other genera, for example, monolaurin suppressed growth of vancomycin- resistant

Enterococcus faecalis in the presence of vancomycin and blocked the induction of vancomycin resistance (Ruzin and Novick, 1998).

A detailed study by Ruzin and Novick (2000) showed that ML inhibited the production of exoenzymes and virulence factors in *Staph. aureus* at concentrations that did not inhibit bacterial growth. Moreover, they were able to show rapid hydrolysis of ML with liberation of lauric acid (*t*1/2 of ~5 min) in the presence of *Staph. aureus* cells. These findings led them to suggest that the prolonged inhibitory effects associated with ML might be due to liberation of lauric acid by hydrolases present in the cell preparation. They were not, however, able to rule out the possibility that ML itself might have some inhibitory action prior to its hydrolysis. These findings raise important questions for fatty acid derivatives of carbohydrates. It is not clear, for example, whether fatty acid esters of carbohydrates are similarly hydrolysed or whether their mode of action might depend on such hydrolysis.

Previous studies in this laboratory, using *Listeria* strains, showed that an ether analog of a CFA retained higher or similar antimicrobial activity to the ester derivative, indicating that the release of a free fatty acid was not an absolute requirement for antimicrobial activity (Nobmann *et al.*, 2009). The analysis of both ester and ether fatty acid derivatives of the same carbohydrate, as well as both alpha and beta configurations of the carbohydrate moiety, suggested that the carbohydrate moiety might also be involved in the antimicrobial activity of fatty acid derivatives (Nobmann *et al*., 2009).

The current study assessed the antimicrobial efficacy of these novel carbohydrate fatty acid derivatives against a range of *Staph. aureus* strains, as well as further investigating the mechanisms of action of the range of compounds to assess relationships between the defined structural differences and the mode of action. Antimicrobial efficacy was evaluated by comparison of MIC and effects on cell lag time, while more detailed studies on how the

compounds might work included membrane permeability studies monitoring absorbance at 260nm, BacLight live/dead assay and cell viability using BacTiter Glo assay.

2. MATERIALS AND METHODS

2.1 Bacteria and culture conditions

Bacterial strains used in this study are listed in Table 1. Stock cultures were maintained in tryptic soy broth (TSB, Scharlau Chemie, Spain) supplemented with 20% glycerol at -70°C. For use in experiments, working cultures were grown routinely by subculturing a loop full of stock culture into TSB tubes and incubating at 35°C for 18 h.

2.2 Test compounds

Lauric acid (LA), caprylic acid (CA), and their corresponding monoglycerides, monolaurin (ML) and monocaprylin (MC) (Sigma-Aldrich ~99% purity), were used as standards for comparison against the CFA derivatives. Nisin, from *Lactococcus lactis* 2.5% (≥1.000.000 per IU/g), was purchased from Sigma, and was used as a positive control compound for membrane disruption studies. Carbohydrate fatty acid derivatives used in this study (Figure 1) were synthesized according to Smith *et al.*, 2008. Stock solutions (100 mmol l^{-1}) of test compounds (CFA derivatives and standards) were prepared in sterile hydroalcoholic diluent (ethanol-distilled water, 1:1) and stored at -20°C. Stock solutions were diluted in TSB or phosphate-buffered saline solution (PBS; pH 7.4, Sigma) to obtain working concentrations.

2.3 Minimal inhibitory concentration (MIC)

MICs were determined using a broth microdilution assay as previously described (Nobmann *et al.*, 2009). Briefly, serial dilutions of each compound were prepared in sterile TSB to a final volume of 100µl in 96-well microtiter plates (Sarstedt Ltd.). Each well was inoculated with 100µl

of the test organism in TSB to a final concentration of approximately $1x10^6$ cfu ml^{-1}. The MIC was taken as the lowest concentration of test compound at which growth was inhibited after 24 h of incubation at 35°C. Controls included: a) uninoculated media without test compound to assess changes in the media; b) uninoculated media containing the test compound to assess background noise; c) inoculated media without test compound to evaluate the microbial growth under optimal conditions; d) inoculated media without test compound but containing corresponding amount of ethanol to account for a possible antagonist or synergistic activity of the alcohol used in the preparation of the test compound.

2.4 Increase in Lag time (Δλ)

The increase in Lag time was calculated using data from absorbance based broth microdilution assays using Gen5™ software. The Δλ was defined as the time required for the culture with test compound to record an increase in OD_{600} of 0.10 minus the time that the culture without compound, took to reach the same increase in OD_{600}.

2.5 Effect of antimicrobial compounds on the cell membrane

For the mode of action studies, the laurate glucopyranoside derivatives (compound **1** to **4**) were selected. These are a closely related group of compounds which differ by a single structural variable, and were evaluated in order to asses relationships between structural differences *e.g.* glycoconjugate linkage and anomeric configuration and antimicrobial efficacy.

2.5.1 Leakage of 260-nm-absorbing material

Bacterial strains were cultured in TSB and incubated at 35°C for 18 h. After incubation, bacteria were harvested by centrifugation at 10,000 *g* for 10 min at 4°C, the supernatant was discarded and the cells were washed twice with PBS; pH 7.4. Suspensions were adjusted to achieve a bacterial concentration of approximately 10^9 cfu ml^{-1}. The CFA derivatives were added to bacterial suspensions at 4X MIC. Suspensions were incubated in a water bath at 35°C. Samples

of 1.5 ml were removed at time 0, and after 15, 30, 45, 60 and 120 minutes; centrifuged at 10,000 *g* for 10 minutes at 4°C. 200 µl of supernatant for each treatment was added to the wells of a 96-well plate (UV–transparent flat-bottom microplates, Costar Cat. No. 3635) and absorbance values at 260 nm were recorded using a UV spectrophotometer (Synergy HT, Bio-Tek). The following controls were included: a bacterial suspension in sterile PBS without antimicrobial agents as the negative control; a fatty acid control (inoculated PBS containing LA); a positive control: Nisin and finally a monoglyceride control: Monolaurin. Where applicable, independent readings were also taken, in the presence of antibacterial agents only, to enable corrections for background contributions.

2.5.2 Modified Live/Dead BacLight assay

The Live/Dead® BacLight viability kit from Molecular Probes, Inc. (Eugene, Oreg.) was used as described by Hilliard *et al*., (1999). In this assay, the SYTO-9 and propidium iodide stains compete for binding to the bacterial nucleic acid. SYTO-9 labels cells with both damaged and intact membranes, whereas propidium iodide penetrates only cells with damaged membranes.

A culture of *Staph. aureus* ATCC 25923 as a model organism was grown to late log phase in 30 ml of TSB. 25 ml of the bacterial culture were concentrated by centrifugation at 10,000 *g* for 10 minutes. The supernatant was removed and the pellet was washed once in filter-sterilized distilled water and resuspended to 1/10th of the original volume and then diluted 1:20 into either sterile de-ionized water (for live bacteria estimation), 70% isopropyl alcohol (for dead bacteria estimation) or sterile de-ionized water containing test compounds at 4x MIC.

Different proportions of the live and dead cells were mixed to obtain cell suspensions containing five different ratios, i.e. 100:0, 75:25, 50:50, 25:75 and 0:100 (in %), of live and dead cells for a data set to provide a standard curve.

Bacterial and treatment suspensions were incubated at room temperature for one hour. At the end of the incubation period, the suspensions were centrifuged at 10,000 *g* for 10 min, washed once in sterile de-ionized water, and resuspended to achieve 2×10^{7} bacteria ml^{-1}.

A volume of 100 µl of each bacterial or treatment suspension was added in triplicate into separate wells of a 96-well microplate (Nunc, black). A 1X stain solution was prepared mixing component A (3.34 mmol l^{-1} SYTO 9 dye) and component B (20 mmol l^{-1} propidium iodide) in equal proportion. 100 µl of 1X stain solution was then added to each well, and the plate was incubated in the dark for 15 min at room temperature. At the end of the incubation period, with the excitation wavelength at 485 nm, the fluorescence intensity of SYTO-9 was measured at 530 nm (emission 1; Green) for each well. With the excitation wavelength still centered at 485 nm, the fluorescence intensity of propidium iodide was measured at 630 nm (emission 2; Red) for each well of the entire plate. The Green/Red (G/R) ratio was obtained by dividing the fluorescence intensity of the stained bacterial suspensions (F cell) at emission 1 by the fluorescence intensity at emission 2.

G/R Ratio = F cell, emission1/F cell, emission 2

The Ratio G/R was plotted versus percentage of live cells in the *Staph. aureus* suspension.

2.5.3 BacTiter-Glo™ assay.

The BacTiter-Glo™ Microbial Cell Viability assay is a method for determining the number of viable bacterial cells in a culture based on quantifying the ATP present, as an indicator of metabolic activity. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of cells in culture.

An overnight culture of *Staph. aureus* ATCC 25923 in Mueller Hinton (MH) broth was diluted 100-fold in fresh MH broth and used as inoculum. CFA derivatives were used at a concentration of 4X MIC. Each well of a 96-well microtiter plate contained 100µl of the

inoculum and 100µl of the CFA derivatives. Control wells containing medium without cells were prepared to obtain a value for background luminescence. Cells without compound were used as an ATP positive control. The microtiter plate was incubated at 35°C for 5 hours. One hundred microliters of the culture was taken from each well, and mixed with the same volume of the BacTiter-Glo™ reagent in a white opaque-walled microtiter plate (Nunc). Plates were incubated for five minutes and luminescence was recorded in a multi-detection microplate reader (Synergy HT, Bio-Tek).

2.6 Statistical analysis

All experiments were performed in duplicate and replicated at least three times. Statistical differences between compound efficacies were determined using ANOVA followed by LSD testing at $p < 0.05$ level using SPSS software, Version 15.

3. RESULTS

3.1 Minimum inhibitory concentration

The MICs of the CFA derivatives for the five *S. aureus* strains tested are shown in Table 2. The concentration of ethanol in the control wells corresponding to the concentration of ethanol in the test wells had no independent inhibitory effect on bacterial growth. Caprylic acid was the least effective of all compounds tested ($p < 0.05$), with MICs of 10 mmol l^{-1} or greater for all bacteria. In contrast, lauric acid had MICs eight- to 16-fold lower than those of caprylic acid. Monolaurin and monocaprylin showed greater activity ($p<0.05$), with values between 0.04-0.08 mmol l^{-1} and 2.5-5 mmol l^{-1} respectively, than their corresponding free fatty acids (LA, CA) with values between 0.63-1.25 mmol l^{-1} and 10-20 mmol l^{-1}. Compounds **2** (α-glucose lauric ether), **3** (β-glucose lauric ester) and **6** (α-mannose lauric ester) were the most active carbohydrate fatty acid

derivatives with MIC values between 0.04-0.16 mmol l^{-1}, comparable to ML (Table 2). The next in order of overall efficacy was compound **1** (α-glucose lauric ester), with MICs between 0.08 mmol l^{-1} and 1.25 mmol l^{-1} with a difference between strains observed. The antimicrobial activity of compound **4** (β-glucose lauric ether) was significantly lower than that observed with the corresponding α-glucose lauric ether (compound **2**, $p < 0.05$), but additionally for compound (**4**) in particular, strain differences were observed (Table 2). Compound **5** had an activity comparable to MC.

3.2 Increase in Lag time for *Staph. aureus*

Where appropriate, the increase in lag time was estimated for *Staph. aureus* ATCC 25923 in the presence of a range of CFA derivatives. For the concentrations at which the compounds were less effective (sub MIC concentrations), an increase in the lag time could be calculated to allow comparison between compounds and concentrations. The effect was found to be concentration and compound dependent, with a major effect at concentrations close to the MIC values (Table 3) ($p < 0.05$). For example, at 2-fold sub-MIC concentrations, the fatty acids (LA, CA) and monoglycerides (ML, MC) standards, as well as compound **1** and compound **5**, showed a significant increase in lag time of approximately two to six hours compared to that of the cultures without treatment. At a 4-fold sub-MIC concentration, a relatively small increase was observed. For compound **4**, a different trend was detected, with a more gradual increase in lag time with an effect observed even at 8-fold sub-MIC concentrations (from 1.25 to 0.16 mmol l^{-1}).

3.3 Leakage of material absorbing at 260nm

Nucleic acid and its related compounds, such as pyrimidines and purines, absorb UV light at a wavelength of 260 nm. The presence of these materials in a suspension may be used as an indicator of damage to the cell membrane. Leakage was determined using nisin as a control, a

compound that is known to cause membrane damage. Table 4 shows the increase of OD_{260} for all the strains and compounds tested after 120 min of exposure. The five bacterial strains showed similar patterns in their release of material absorbing at 260 nm. The amount of UV absorbing substances released increased as the time of exposure increased, when *S. aureus* suspensions were treated with nisin, LA, ML and CFA derivatives at 4x MIC. The OD_{260} increased rapidly at first, and the absorbance values continued to increase, but more gradually after 60 minutes. An exception was observed for compound **4**, β-glucose lauric ether, which even after 120min of exposure did not display a significant increase in OD260 nm.

3.4 Live/Dead BacLight assay

Bacterial membrane damage was further assessed by using the Live/Dead® BacLight viability assay. The fluorescence intensities of the stained bacterial suspension at 535nm (G, green) and 615nm (R, red) represent live and dead cells, respectively. The fluorescence G/R ratio, obtained by dividing the green and red intensities, were plotted against the live:dead cells ratio used for the standard curve. Results are shown in Table 5.

Exposure of staphylococcal cells to CFA derivatives (at 4x MIC for one hour) showed altered cytoplasmic membrane permeability for lauric acid and the alpha compounds. Both alpha lauric ester (compound **1**) and the alpha lauric ether (compound **2**), had a low fluorescence ratio, corresponding to 10-20% viability compared to the untreated control.

On the other hand, the beta lauric ester (compound **3**) derivative showed a higher fluorescence ratio, corresponding to retention of approximately 75% viability. The beta ether derivative (compound **4**) did not significantly affect the fluorescence ratio, which was in agreement with the observation for the leakage studies at 260nm for this compound.

3.5 BacTiter Glo assay

The luminescence signal is proportional to the amount of ATP present, which is directly proportional to the number of metabolically active cells in the culture. The results are shown in Figure 2. The alpha lauric derivatives (compounds **1** and **2**) resulted in a noticeable decrease in ATP levels. For the lauric acid and the beta derivatives (compounds **3** and **4**), there was also a decline in ATP detected, but to a lesser extent than that observed with the alpha lauric derivatives. Again, this reinforces the importance of the anomeric configuration in antimicrobial efficacy.

4. DISCUSSION

The emergence of methicillin-resistant *Staph. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and extended spectrum β-lactamase-producing Enterobacteriaceae has seriously reduced the number of empirical agents suitable for selected indications (Isturiz, 2008). Natural products, either as pure compounds or as standardized extracts, may provide opportunities for new drug leads because of the unmatched availability of chemical diversity (Cos *et al*., 2006).

Previous work in this laboratory showed that novel CFA derivatives were active against Gram positive bacteria, especially the foodborne pathogen *L. monocytogenes* (Nobmann *et al*., 2009). Thus the present study evaluated the antimicrobial properties of pure novel carbohydrate fatty acid esters and their corresponding ether analogs, against five *Staph. aureus* strains including MRSA strains.

Ruzin and Novik, (2000) showed that monolaurin was rapidly hydrolysed ($t_{1/2}$ of ~5 min) by esterases in *Staph. aureus* cultures suggesting that inhibitory activity could be due to free fatty acid liberated from monolaurin by hydrolysis. Isaacs and Lamp (2000) suggested that changing the linkage in a monoglyceride between the fatty acid and the glycerol backbone from an ester

bond to an ether linkage could increase the antimicrobial activity of some medium-chain monoglycerides. Here, we have examined antimicrobial activity using pure CFA derivatives whose carbohydrate moieties were effectively "locked" in either the alpha or beta configuration. These compounds also differed in the type of fatty acid carbohydrate linkage (ester and ether conjugates), and the length of fatty acid chain (lauric and caprylic acid).

The CFA derivatives showed various antimicrobial activities against a panel of Gram-positive bacteria. In the data presented here we show that there is little difference in antibacterial activity between a fatty acid derivative of alpha methyl glucoside whether the linkage is via an ester or an ether bond (compound **1** and **2**). This finding suggests that the hydrolysis of the bond between the fatty acid and the carbohydrate is not required for antimicrobial activity, as the ether linkage is not readily hydrolysed by esterases. However, an alternative explanation whereby the antimicrobial activity of the fatty acid ether derivative is due to binding at a site different to that of the ester derivative cannot be discounted. This explanation would, however, have to postulate two sites of action for these compounds, both equally bactericidal, which seems unlikely.

What is surprising is that antimicrobial activity is not sensitive to the presence or absence of the bulky carbohydrate group but is sensitive to the configuration of the anomeric carbon of the carbohydrate moiety. Despite their similarity in structure, compound **2** (α-glucose lauric ether), was far more active against most *Staph. aureus* strains than compound **4** (β-glucose lauric ether). This observation is in agreement with Watanabe *et al*. (2000) who found that configuration of the hydroxyl group in the carbohydrate moiety markedly affected the antibacterial activity.

The study of lag time increase showed that sub-MIC concentrations of CFAs can modify bacterial growth significantly. Antimicrobial efficacy of compound **4** was considerably lower than that of the other derivatives, nevertheless, these results show that there is a significant effect

of this compound on bacterial growth, even at sub-MIC concentrations. This is important in light of its potential combination with other antimicrobials for optimization of application of CFA derivatives. It is also possible that these antimicrobial agents might also work synergistically with certain antibiotics as has been previously reported by Ved *et al*., (1990), who showed that dodecylglycerol and penicillin G acted synergistically to decrease the MIC of both compounds towards Gram positive bacteria. Haynes *et al.* (1994) observed that the alkyl glycerol, ether rac-1-O-dodecylglycerol, inhibited the growth of members of two genera of yeasts, *Candida* and *Cryptococcus*, and was strongly synergistic with amphotericin B. Similarly, Rouse *et al*., (2005) demonstrated that mupirocin and monolaurin formulations were active in vitro against *Staph. aureus*. Preuss *et al*.(2005), found that Origanum oil, carvacrol, and other essential oils, combined with monolaurin killed *Staph. aureus in vitro*, and proposed that origanum and/or monolaurin, alone or combined with antibiotics, might prove useful in the prevention and treatment of severe bacterial infections.

All of the CFA derivatives studied here, except for compound **4**, had the ability to affect the cytoplasmic membrane of *Staph. aureus* as judged by the leakage of 260-nm-absorbing material assay and the BacLight Live/Dead fluorescence assay; however, derivatives with different structures had different concentration-activity profiles.

In contrast to the leakage at 260nm and the BacLight data, that indicated no membrane damage, compound **4** appreciably had an effect on bacterial growth as seen by the increase in lag time and decrease of ATP levels, suggesting that this compound could have a different mechanism of action than membrane damage. The BacLight Live/Dead fluorescence and the ATP luminescence assays focus on the viability of the microorganism, but from different perspectives, the first one assesses the permeability of the cell membrane and the second one involves the metabolic state of the cell. For example, compound **3**, which had a high

antimicrobial activity, showed low ability to permeabilize the membrane according to the Live/Dead fluorescence assay, but showed a decrease in the metabolic activity according to the ATP luminescence assay. These results suggest that the CFA derivatives might have different mechanisms or sites of action that do not only involve membrane damage.

All CFA derivatives had an effect on methicillin-resistant *Staph. aureus* to an extent similar to that of the nonresistant strains, suggesting that the mechanisms of methicillin resistance did not affect the CFA derivatives efficacy.

5. CONCLUSIONS

A range of CFA derivatives were shown to have useful antimicrobial activity against *Staph. aureus* strains, with compound efficacy related to structural differences. Carbohydrate anomeric configuration and fatty acid chain length have significant effects on anti-microbial efficacy. Carbohydrate fatty acid derivatives had the ability to affect the cytoplasmic membrane of *Staph. aureus* and MRSA strains and may account for at least a component of the mode of action of these compounds. Further insight into the mechanism of action will aid in the production of CFA derivatives as possible future therapeutic antimicrobial compounds.

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Table 1. *Staphylococcus aureus* strains used in this study.

Strain	**Reference**	**Source / Comments**
Staph. aureus	ATCC 25923	clinical isolate
Staph. aureus	NCTC 1803	mammal, ovine gangrenous mastitis
Staph. aureus	ATCC 33591	Methicillin resistant
Staph. aureus	ATCC 33592	Blood, Gentamicin- and methicillin-resistant
Staph. aureus	ATCC 43300	clinical isolate, F-182, Methicillin- and oxacillin-resistant

Table 2. Minimum Inhibitory Concentration (MIC) values of CFA derivatives and standards.

Microorganism	Fatty acid		Monoglyceride		Carbohydrate fatty acid derivatives					
	LA	**CA**	**ML**	**MC**	**1**	**2**	**3**	**4**	**5**	**6**
S. aureus ATCC 25923	0.63	10	0.04	5	0.31	0.04	0.04	2.5	2.5	0.04
S. aureus NCTC 1803	0.63	10	0.04	2.5	0.31	0.04	0.08	1.25	1.25	0.04
S. aureus ATCC 33591	1.25	10	0.04	2.5	1.25	0.04	0.04	2.5	5	0.04
S. aureus ATCC 33592	1.25	20	0.04	2.5	0.08	0.08	0.04	5	2.5	0.16
S. aureus ATCC 43300	1.25	10	0.08	2.5	0.08	0.08	0.04	10	2.5	0.08

MIC was recorded as the concentration (mmol l^{-1}) that resulted in total inhibition of all replicates after 24h at 35℃. LA: Lauric acid; CA: Caprylic acid; ML: Monolaurin; MC: Monocaprylin 1. Methyl 6-O-lauroyl-α-D-glucopyranoside; 2. Methyl 6-O-dodecanyl-α-D-glucopyranoside; 3. Methyl 6-O-lauroyl-β-D-glucopyranoside; 4. Methyl 6-O-dodecanyl-β-D-glucopyranoside; 5. Methyl 6-O-octanoyl-α-D-glucopyranoside; 6. Methyl 6-O-lauroyl-α-D-mannopyranoside

Table 3. Increase in Lag time (Δλ) for *Staph. aureus* ATCC 25923 in the presence of standards and CFA derivatives.

***S. aureus* ATCC 25923**					
Compound	**(mmol l^{-1})**	**Δ λ (h) SD**	**Compound**	**(mmol l^{-1})**	**Δ λ (h) SD**
LA	0.04	0.0 ± 0.000	**CA**	0.63	0.0 ± 0.000
	0.08	0.0 ± 0.000		1.25	0.2 ± 0.009
	0.16	0.5 ± 0.364		2.5	0.6 ± 0.221
	0.31	4.2 ± 0.898		5	3.0 ± 0.312
	0.63	N.G		10	N.G
ML	0.02	3.9 ± 0.684	**MC**	1.25	0.0 ± 0.000
	0.04	N.G		2.5	1.8 ± 0.986
				5	N.G
1	0.04	0.0 ± 0.000	**5**	0.63	0.5 ± 0.327
	0.08	0.7 ± 0.167		1.25	1.3 ± 0.376
	0.16	6.1 ± 0.595		2.5	N.G
	0.31	N.G			
4	0.16	2.7 ± 1.777			
	0.31	3.9 ± 2.865			
	0.63	4.1 ± 2.021			
	1.25	6.4 ± 1.615			
	2.5	N.G			

Values are expressed as the difference between the treated culture and culture without compound. SD: Standard deviation. N.G.: No growth. LA: Lauric acid; CA: Caprylic acid; ML: Monolaurin; MC: Monocaprylin; 1. Methyl 6-O-lauroyl-α-D-glucopyranoside; 4. Methyl 6-O-dodecanyl-β-D-glucopyranoside; 5. Methyl 6-O-octanoyl-α-D-glucopyranoside.

Table 4. Effects of CFAs at 4x MIC on membrane integrity in *S. aureus* strains measured by release of UV absorbing components at 260 nm.

Organism	Time (mins)	Negative control		Positive control (Nisin)		Free fatty acid (Lauric acid)		Monoglyceride (Monolaurin)		Carbohydrate fatty acid derivatives							
										1		2		3		4	
S. aureus	0	0	SD	0	SD	0	SD	0	SD	0	SD	0	SD	0	SD	0	SD
ATCC	15	0.019	±0.006	0.169	±0.054	0.120	±0.005	0.180	±0.088	0.107	±0.021	0.106	±0.029	0.149	±0.046	0.037	±0.033
25923	30	0.033	±0.019	0.257	±0.055	0.214	±0.078	0.201	±0.042	0.155	±0.021	0.155	±0.020	0.220	±0.082	0.045	±0.027
	45	0.050	±0.031	0.321	±0.061	0.264	±0.038	0.226	±0.043	0.208	±0.039	0.189	±0.013	0.299	±0.036	0.056	±0.037
	60	0.098	±0.062	0.410	±0.080	0.255	±0.024	0.272	±0.078	0.240	±0.037	0.234	±0.033	0.315	±0.074	0.062	±0.040
	120	0.073	±0.004	0.396	±0.012	0.334	±0.040	0.291	±0.003	0.415	±0.010	0.355	±0.015	0.408	±0.031	0.088	±0.007
S. aureus	0	0		0		0		0		0		0		0		0	
NCTC	15	0.008	±0.009	0.023	±0.010	0.138	±0.005	0.144	±0.006	0.087	±0.009	0.058	±0.020	0.156	±0.005	0.001	±0.000
1803	30	0.029	±0.012	0.077	±0.006	0.176	±0.009	0.172	±0.009	0.164	±0.082	0.141	±0.029	0.204	±0.008	0.004	±0.005
	45	0.048	±0.001	0.166	±0.023	0.308	±0.001	0.344	±0.004	0.267	±0.016	0.210	±0.021	0.289	±0.021	0.018	±0.004
	60	0.049	±0.002	0.190	±0.013	0.239	±0.020	0.267	±0.005	0.277	±0.009	0.254	±0.001	0.274	±0.002	0.003	±0.004
	120	0.084	±0.009	0.256	±0.011	0.288	±0.068	0.309	±0.008	0.323	±0.012	0.316	±0.019	0.305	±0.006	0.001	±0.000
S. aureus	0	0		0		0		0		0		0		0		0	
ATCC	15	0.007	±0.004	0.127	±0.006	0.073	±0.012	0.125	±0.039	0.083	±0.046	0.065	±0.005	0.110	±0.012	0.006	±0.006
33591	30	0.016	±0.014	0.152	±0.036	0.066	±0.010	0.162	±0.033	0.138	±0.069	0.127	±0.020	0.155	±0.050	0.013	±0.013
	45	0.040	±0.007	0.198	±0.050	0.149	±0.016	0.204	±0.060	0.163	±0.072	0.191	±0.027	0.287	±0.110	0.043	±0.034
	60	0.047	±0.046	0.239	±0.100	0.095	±0.045	0.311	±0.114	0.219	±0.102	0.233	±0.076	0.315	±0.131	0.036	±0.040
	120	0.033	±0.003	0.244	±0.019	0.073	±0.005	0.439	±0.007	0.248	±0.004	0.206	±0.013	0.270	±0.010	0.015	±0.003
S. aureus	0	0		0		0		0		0		0		0		0	
ATCC	15	0.011	±0.009	0.101	±0.013	0.137	±0.017	0.178	±0.055	0.187	±0.074	0.162	±0.042	0.266	±0.071	0.019	±0.023
33592	30	0.032	±0.032	0.164	±0.006	0.180	±0.051	0.264	±0.021	0.278	±0.081	0.228	±0.050	0.315	±0.037	0.049	±0.034
	45	0.043	±0.043	0.206	±0.011	0.134	±0.014	0.275	±0.001	0.342	±0.013	0.266	±0.004	0.372	±0.008	0.002	±0.012
	60	0.043	±0.043	0.249	±0.006	0.201	±0.028	0.290	±0.017	0.344	±0.078	0.303	±0.044	0.419	±0.015	0.015	±0.038
	120	0.096	±0.096	0.352	±0.127	0.237	±0.008	0.314	±0.038	0.399	±0.070	0.352	±0.053	0.473	±0.069	0.013	±0.022
S. aureus	0	0		0		0		0		0		0		0		0	
ATCC	15	0.010	±0.007	0.109	±0.005	0.135	±0.002	0.352	±0.006	0.180	±0.083	0.138	±0.068	0.160	±0.057	0.056	±0.048
43300	30	0.031	±0.019	0.180	±0.011	0.167	±0.003	0.488	±0.009	0.323	±0.114	0.265	±0.092	0.314	±0.085	0.047	±0.037
	45	0.030	±0.003	0.242	±0.020	0.225	±0.012	N.D		0.336	±0.004	0.255	±0.009	0.336	±0.002	0.020	±0.006
	60	0.029	±0.006	0.248	±0.043	0.236	±0.006	0.528	±0.002	0.422	±0.096	0.343	±0.045	0.411	±0.068	0.042	±0.035
	120	0.050	±0.031	0.289	±0.130	0.416	±0.044	0.542	±0.009	0.507	±0.036	0.415	±0.027	0.494	±0.036	0.039	±0.040

1. Methyl 6-O-lauroyl-α-D-glucopyranoside; 2. Methyl 6-O-dodecanyl-α-D-glucopyranoside; 3. Methyl 6-O-lauroyl-β-D-glucopyranoside; 4. Methyl 6-O-dodecanyl-β-D-glucopyranoside; 6. Methyl 6-O-lauroyl-α-D-mannopyranoside. N.D: not determined, SD: Standard Deviation

Table 5. Effect of CFA derivatives at 4x MIC on membrane integrity in *Staph. aureus* ATCC 25923 measured by the BacLight assay.

Antimicrobial compounds	**Live %**	**SD**
None (non treated culture)	100	
Nisin (positive control)	8.36	± 3.00
Lauric acid	8.39	± 1.66
Alpha lauric ester (**1**)	17.38	± 5.41
Alpha lauric ether (**2**)	16.34	± 3.13
Beta lauric ester (**3**)	76.05	± 13.62
Beta lauric ether (**4**)	147.85	± 48.93

Values are expressed as percentage of those obtained with control cultures not exposed to the antimicrobial compounds. SD: Standard deviation. 1. Methyl 6-O-lauroyl-α-D-glucopyranoside; 2. Methyl 6-O-dodecanyl-α-D-glucopyranoside; 3. Methyl 6-O-lauroyl-β-D-glucopyranoside; 4. Methyl 6-O-dodecanyl-β-D-glucopyranoside.

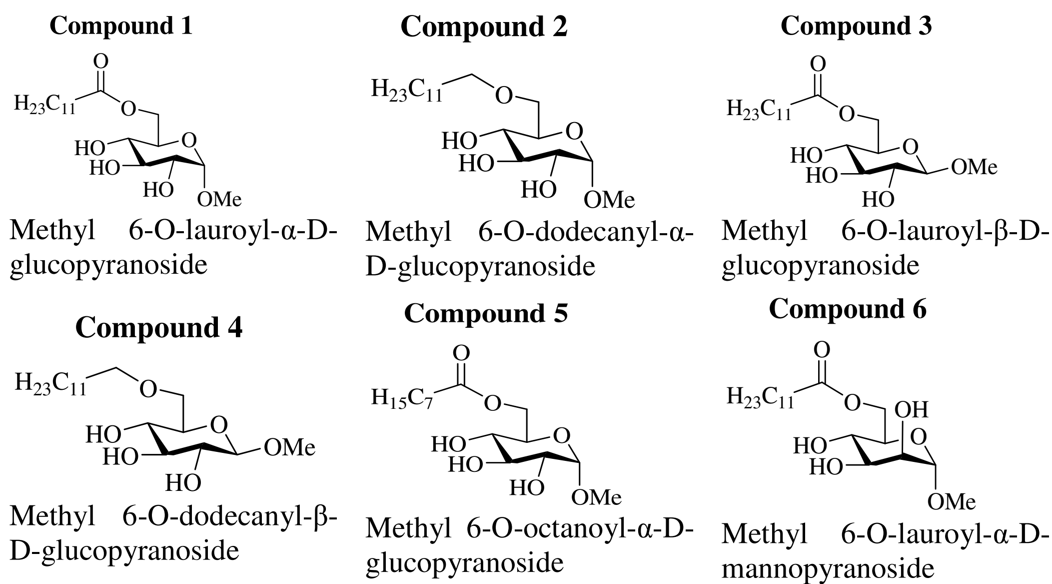

Figure 1. Structures of the novel carbohydrate fatty acid derivatives investigated for antimicrobial activity.

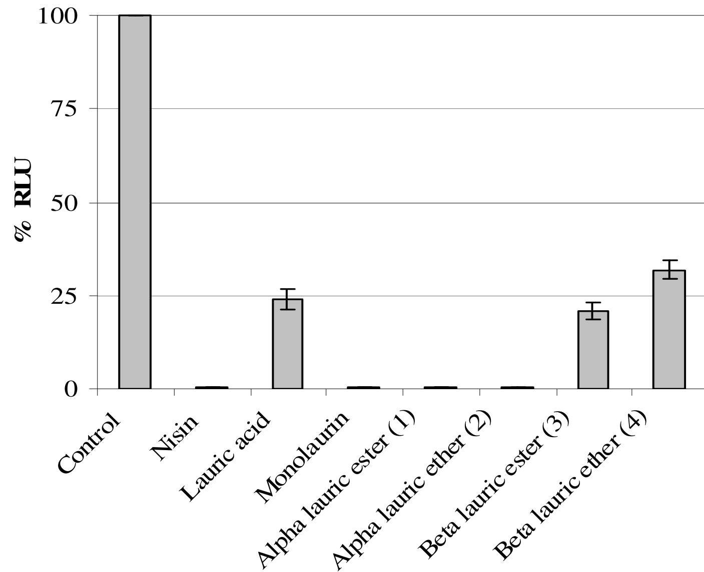

Figure 2. Relative luminescence activity of *Staph. aureus* ATCC25923 cells in response to CFA derivatives using the BacTiter-Glo™ assay.

Values are expressed as percentage of those obtained with control cultures not exposed to the antimicrobial compounds. 1. Methyl 6-O-lauroyl-α-D-glucopyranoside; 2. Methyl 6-O-dodecanyl-α-D-glucopyranoside; 3. Methyl 6-O-lauroyl-β-D-glucopyranoside; 4. Methyl 6-O-dodecanyl-β-D-glucopyranoside. Error bars represent Standard deviation values of replicate experiments.