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The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms

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ABSTRACT

Novel mono-substituted carbohydrate fatty acid (CFA) esters and ethers were investigated for their antibacterial activity against a range of pathogenic and spoilage bacteria focussing on *Listeria monocytogenes*. Carbohydrate derivatives with structural differences enable comparative studies on the structure/activity relationship for antimicrobial efficacy and mechanism of action. The antimicrobial efficacy of the synthesized compounds was compared with commercially available compounds such as monolaurin and monocaprylin, as well as the pure free fatty acids, lauric acid and caprylic acid, which have proven antimicrobial activity. Compound efficacy was compared using an absorbance based broth microdilution assay to determine the minimum inhibitory concentration (MIC), increase in lag phase and decrease in maximum growth rate. Among the carbohydrate derivatives synthesized, lauric ether of methyl α-D-glucopyranoside and lauric ester of methyl α-D-mannopyranoside showed the highest growth-inhibitory effect with MIC values of 0.04 mM, comparable to monolaurin. CFA derivatives were generally more active against Gram positive bacteria than Gram negative bacteria. The analysis of both ester and ether fatty acid derivatives of the same carbohydrate, in tandem with alpha and beta configuration of the carbohydrate moiety suggest that the carbohydrate moiety is involved in the antimicrobial activity of the fatty acid derivatives and that the nature of the bond also has a significant effect on efficacy, which requires further investigation. This class of CFA derivatives has great potential for developing antibacterial agents relevant to the food industry, particularly for control of *Listeria* or other Gram-positive pathogens.

1. Introduction

Consumer demand for fresh, minimally processed and "natural" foods, along with the requirement for maintenance and enhancement of safety, quality and shelf-life characteristics has fuelled research for alternative antimicrobials. *Listeria monocytogenes* has emerged as one of the most important food pathogens in ready-to-eat processed meals and dairy foods (EFSA, 2007), given that it can adapt to a wide range of food processes and storage conditions including refrigeration temperatures, and acidic or high salt foods. Moreover, *Listeria* has one of the highest case fatality rates of all the foodborne infections: 20–30% (De Valk et al., 2005). Therefore, there is a need for investigation of new approaches for the control or elimination of this pathogen in foods whilst also addressing food spoilage concerns.

Fatty acids (FA) and their corresponding esters are one group of chemicals found in nature considered to have little or no toxicity, with proven antimicrobial activity. Kabara et al. (1972) showed that while fatty acids esterified with monohydric alcohols were inactive against microorganisms, those esterified with certain polyhydric alcohols yielded antimicrobial derivatives (Conley and Kabara, 1973). Monoglycerides (MG) are commonly employed in the food industry as flavoring and emulsifying agents and Monolaurin (ML), a food-grade glycerol monoester of lauric acid, is approved in the US as a food emulsifier (21 CFR GRAS 182.4505). The anti-listerial activity of fatty acids and monoglycerides has been previously documented (Oh and Marshall, 1993; Wang and Johnson, 1997; Sprong et al., 2001). Their antimicrobial activity against spoilage microorganisms has also been reported (Ouattara et al., 1997; Blaszyk and Holley, 1998).

Sugar esters are biodegradable, nontoxic and nonionic surfactants, currently employed in the food, pharmaceutical, cosmetics and detergent industries (Hill and Rhode, 1999; Piccicuto et al., 2001). Furthermore, their antimicrobial activities have been reported (Monk et al., 1996; Devulapalle et al., 2004; Ferrer et al., 2005).

Carbohydrate fatty acid (CFA) esters have been synthesized chemically and enzymatically by interesterification, transesterification and direct esterification. An issue regarding the synthesis of commercial sucrose esters is related to the high functionality of the carbohydrate molecule with many hydroxyl groups, which compete during the derivatization step, leading to product mixtures of mono-, di- and polyesters (Hill and Rhode, 1999). Enzymatic synthesis of novel sugar fatty acid esters has been widely employed and can be highly regioselective, although for some carbohydrates minor regiomeric isomers may be obtained.

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The exact mode of action of fatty acid esters has not yet been elucidated, but the cytoplasmic membrane is thought to be the primary site of action for fatty acid esters, affecting respiratory activity through inhibition of enzymes involved in oxygen uptake (Kabara, 1993). Ruzin and Novick (2000) reported a monolaurin esterase activity in association with the *S. aureus* cell membrane and cytoplasm. It was shown that the half life of monolaurin in cultures of *S. aureus* was *ca.* 5 min due to its cleavage by cellular esterases. These studies raise the question as to whether the ester, or free fatty acid derived from hydrolysis of the ester, was responsible for antimicrobial activity.

Recently, a number of novel fatty acid derivatives of carbohydrates have been synthesized and their antimicrobial activity assessed (Devulapalle et al., 2004; Ferrer et al., 2005). These workers have pointed out that a complication of some earlier studies was that they were carried out using commercial preparations that contained a mixture of compounds. Thus, it was difficult to correlate antimicrobial activity with chemical structure. It is clear that future studies in this area will require the use of pure compounds. Moreover, there is a need to standardize antimicrobial activity of novel compounds by the use of reference compounds. Finally, quantification of antimicrobial activity is desirable to allow comparison between different studies.

The objectives of this study were to compare the *in vitro* antimicrobial activity of a range of pure, novel, fatty acid esters with the corresponding fatty acid ethers and commercial fatty acids and monoglycerides to ascertain the role of the free fatty acid in the antimicrobial efficacy. These compounds were compared quantitatively to allow an estimation of the enhancement of the efficacy over the free fatty acids. This work has used a synthesis designed to allow the production of pure, novel regiochemically defined monosaccharide mono-fatty acid esters, and their corresponding ethers. The effect of different carbohydrate scaffolds as well as a non-carbohydrate (pentaerythritol) on antimicrobial efficacy was also examined. The effect of fatty acid chain length and anomeric configuration of the carbohydrate was also explored.

The activity of eight CFA derivatives and three non-carbohydrate polyhydroxylated ester derivatives, together with their corresponding monosaccharide, fatty acids and monoglycerides as controls, were assessed against a range of Gram-positive and negative bacteria of interest to the food industry. Efficacy and structure–activity relationships were assessed by comparing MIC values, the increase in Lag phase and maximum specific growth rate.

2. Materials and methods

2.1. Bacteria and growth conditions

Bacterial strains used in this study are listed in Table 1. Stock cultures were maintained in tryptic soy broth (TSB, Sharlau Chemie, Spain) supplemented with 20% glycerol at −70 °C. Cultures were routinely grown by subculturing 100 µL of stock culture into 9 mL TSB and incubating at 35 °C for 18 h, except for *Pseudomonas* spp. which were incubated at 30 °C. All cultures were then maintained on tryptic soy agar (TSA, Sharlau Chemie, Spain) plates at 4 °C. Working cultures were prepared by inoculating a loop of pure culture into TSB and incubating at the optimum temperature for each strain for 18 h. A bacterial suspension was prepared in saline solution (NaCl 0.85%, BioMérieux, France) equivalent to a McFarland standard of 0.5, using the Densimat photometer (BioMérieux, SA, France), to obtain a concentration of 1×10^8 cfu/mL. This suspension was then serially diluted in TSB to obtain a working concentration of 1×10^6 cfu/mL.

Table 1
Microorganisms used in this study

Strain	Reference[a]	Source
Gram-positive bacteria		
Listeria innocua	NCTC 11288	Cow brain, serotype 6a
Listeria monocytogenes	ATCC 7644	Human
Listeria monocytogenes	NCTC 11994	Cheese, serotype 4b
Listeria monocytogenes	NCTC 7973	Pig mesenteric lymph node
Gram-negative bacteria		
Escherichia coli	ATCC 25922	Clinical isolate
Escherichia coli	NCTC 12900	Human, serotype O157:H7 nontoxigenic
Salmonella enterica (serovar Typhimurium)	ATCC 14028	Animal tissue
Enterobacter aerogenes	ATCC 13048	Sputum
Pseudomonas fluorescens	*	Lettuce

[a] Strains indicated with an asterisk were provided by the Department of Life Sciences, University of Limerick, Ireland.

2.2. Chemical synthesis

Chemical synthesis was performed according to Smith et al. (in press). An overview of the test compounds synthesized and used in the antimicrobial assay is given in Fig. 1.

2.3. Test compounds preparation

The saturated free fatty acids, lauric acid (LA–C_{12}) and caprylic acid (CA–C_8), as well as their corresponding monoglycerides, monolaurin (ML) and monocaprylin (MC) (Sigma-Aldrich ~99% purity), were used as standards in this study.

Stock solutions (100 mM) of test compounds and standards were prepared in sterile hydroalcoholic diluent (ethanol–distilled water, 1:1) and stored at −20 °C. Stock solutions were diluted in TSB to obtain initial working concentrations (10 or 20 mM).

2.4. Antimicrobial activity assay

Solutions of the working test compounds and standards were serially diluted in sterile TSB to a final volume of 100 µL within the 96-well microtiter plate. 100 µL of freshly prepared inoculum of the organism under study was added to each appropriate well. The final concentration of each microorganism in each well was approximately 5×10^5 cfu/mL and the concentration of chemical compounds ranged from 1:2 to 1:256. Each concentration was assayed in duplicate. The following controls were used in the microplate assay for each organism and test compound; blank: uninoculated media without test compound to account for changes in the media during the experiment; negative control: uninoculated media containing only the test compound; positive control 1: inoculated media without compound; positive control 2: inoculated media without compound but including the corresponding sugar to evaluate any effect of the sugar alone; and positive control 3: inoculated media without compound but with the equivalent concentration of ethanol used to dissolve the test compound thereby assessing any activity of the alcohol. The 96-well plates were incubated for 18 h in a microtiterplate reader (PowerWave microplate Spectrophotometer, BioTek) at 35 °C, except for *Pseudomonas* spp. which were incubated at 30 °C, and effects were monitored by measuring the optical density (OD) at 600 nm for each well every 20 min with 20 s agitation before each OD measurement. Each experiment was replicated three times.

2.5. Data analysis

2.5.1. Minimum inhibitory concentration (MIC)

The MIC was defined as the lowest concentration of compound that showed no increase in OD values for all the replicates compared to the negative control after 18 h. The absorbance readings obtained from the kinetic data were plotted against time to obtain the growth curves of the test organisms. Subtraction of the absorbance of the negative control eliminated interferences due to possible variations in the media.

2.5.2. Lag time increase (λ)

The increase in Lag time was calculated using the Gen5™ software. The increase in lag time was defined as the time required for the culture