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Antimicrobial Evaluation of Novel Fatty Acid Derivatives and Other Natural Antimicrobials

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Antimicrobial evaluation of novel fatty acid derivatives and other natural antimicrobials

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ABSTRACT

The food industry has shown increased interest for novel natural antimicrobials due to consumer demand for foods with fewer synthetic additives, increased safety, quality and shelf-life. Concurrently, the emergence of drug resistant bacteria substantiates the need for newer antimicrobial agents. Alternative strategies include the use of novel antimicrobials, such as fatty acid derivatives, essential oils and bacteriocins, with proven antimicrobial properties against a diverse range of bacteria.

This study investigated novel carbohydrate fatty acid (CFA) derivatives for their antibacterial activity against a range of pathogenic and spoilage bacteria. A series of mono-substituted CFA derivatives using lauric and caprylic acids were synthesized. Chemical routes allowing the attachment of the fatty acid as either an ester or ether to the monosaccharide 6-hydroxyl were developed. Structure/activity relationship studies of antimicrobial efficacy allowed some insight into the mechanism of action of these compounds. In addition, selected essential oil (EO) individual components and nisin, both alone and in combination, were also assessed for possible combination strategies with CFA derivatives.

Minimum inhibitory concentration (MIC), IC_{50} values, increase in lag phase and decrease in maximum specific growth rate were determined. Leakage of intracellular material absorbing at 260nm, Live/Dead *Bac*Light fluorescence and ATP BacTiterGlo luminescence assays assessed membrane disrupting effects. Furthermore, a Phenotypic MicroArray assay was used to investigate bacterial cell response in the absence and presence of CFA derivatives.

The laurate ether of methyl α-D-glucopyranoside (**9a**) and laurate ester of methyl α-D-mannopyranoside (**4c**) showed the highest growth-inhibitory effect. CFA

derivatives were significantly more effective against Gram positive 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. Membrane damage was observed and may account for at least a component of the mode of action of these compounds. The ATP assay and phenotypic responses suggested that another mechanism of action might also account for the antimicrobial activity of the beta lauric ether derivative (compound **9b**).

Carvacrol and thymol were found to have the highest antimicrobial activity, followed by citral. Nisin in combination with carvacrol, thymol and citral showed additive effects against *Listeria* spp. The antimicrobial effect of the combination was improved by lowering the pH from neutral to pH6.

CFA derivatives with different structures have great potential for developing antibacterial agents relevant to the food and health care industries. Nisin in combination with individual EO components could lead to useful efficacy as an alternative natural preservative.

Declaration page

I certify that this thesis which I now submit for examination for the award of PhD, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

The Institute has permission to keep, lend or copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged.

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Finally I would like to dedicate this work to my parents and sister. Thanks for your constant support and motivation and for providing an outstanding moral example that has guided me through my life. *Quiero dedicar este trabajo a mis padres y hermana, por brindarme un hogar cálido, por el ejemplo moral proporcionado y por enseñarme que la perseverancia y el esfuerzo son el camino para lograr objetivos. Gracias por su constante estímulo y apoyo en mis estudios.*

SYMBOLS AND ABBREVIATIONS

α	alpha
anhyd	anhydrous
ANOVA	analysis of variance
aq.	aqueous
β	beta
Bn	benzyl
c	concentration
CFA	carbohydrate fatty acid
CFU/mL	colony forming units per millilitre
cm^{-1}	wavenumber
δ	chemical shift in ppm downfield from TMS
°C	degrees Celsius
EDTA	ethylenediaminetetraacetic acid
EO	essential oil
equiv.	equivalents
FA	fatty acid
FIC	fractional inhibitory concentration
g	gram(s)
GRAS	generally recognized as safe
h, min	hour(s), minute(s)
HUS	haemolytic-uremic syndrome
Hz	hertz
J	coupling constant (NMR), in Hz
L	litre
LAB	lactic acid bacteria
NAG	N-acetyl-glucosamine
M	molar
M^+	mass of the molecular ion (mass spectrometry)
MBC	minimum bactericidal concentration
mg	milligram

MG	monoglyceride
MIC	minimum inhibitory concentration
mL	millilitre
mol, mmol	mole, millimole
μM, mM	micromolar, millimolar
MRS	de Man, Rogosa and Sharpe
MRSA	methicillin resistant *Staphylococcus aureus*
ng/μg	nanogram/microgram
NMR	nuclear magnetic resonance
PBS	phosphate buffer solution
PM	phenotype microarray
ppm	parts per million (NMR)
rt	room temperature
TIPS	triisopropylsilyl
TLC	thin layer chromatography
Trityl	triphenylmethyl
TSA	tryptic soy agar
TSB	tryptic soy broth
UV	ultraviolet
VTEC	vero-toxigenic *Escherichia coli*

SOLVENTS AND REAGENTS

AcOH	acetic acid
BnBr	benzyl bromide
Bu_4NI	tetrabutylammonium iodide
CA	caprylic acid
$CHCl_3$	chloroform
CH_2Cl_2	dichloromethane
DMAP	4-Dimethylaminopyridine
DMF	dimethylformamide
EtOAc	ethyl acetate
EtOH	ethanol
H_2	hydrogen gas
HCl	hydrochloric acid
H_2O	water
H_2SO_4	sulfuric acid
KBr	potassium bromide
LA	lauric acid
MeCN	acetonitrile
MeOH	methanol
$MgSO_4$	magnesium sulfate
ML	monolaurin
NaCl	sodium chloride
NaH	sodium hydride
$NaHCO_3$	sodium hydrogen carbonate
NaOH	sodium hydroxide
Py	pyridine
TBAF	tetrabutylammonium fluoride
TBAI	tetrabutylammonium iodine
THF	tetrahydrofuran
TsOH	*p*-Toluenesulfonic acid

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1. INTRODUCTION

This study concerned the synthesis of carbohydrate fatty acid (CFA) derivatives for evaluation as antibacterial agents, with a view to examining the effect of variation of the hydrophilic moiety, the glycoconjugate linkage, and the length of fatty acid chain on antimicrobial activity. Previous studies in the School suggested that the carbohydrate moiety could markedly affect the antimicrobial activity of the fatty acid (Lou, 2005); thus, further investigation was merited. Few studies have attempted to investigate the mechanism of action of these compounds; this work seeks to further knowledge through investigation of structure/activity relationships and mode of action of these compounds.

Fatty acid derivatives are a type of 'natural' compound regarded as non-toxic, bio-compatible and bio-degradable. These types of compounds are of great interest because of their wide variety of applications in the health care and food industries. They are currently used as emulsifiers, non-ionic surfactants, food additives, and even as non-caloric fat substitutes. Sugar esters and monoglycerides have been previously studied by many authors, and antimicrobial activity against a range of bacteria, yeast, fungi and viruses has been demonstrated. Sugar esters are used in Japan as antibacterial agents in canned drinks (Suwa *et al.*, 1988, Moriyama *et al.*, 1996), although in Europe and USA their use in the food industry as a preservative is still limited.

There is potential to modify the carbohydrate fatty acid derivatives' properties by controlling the degree of substitution of the fatty acid and by varying the nature of the fatty acid or the sugar itself.

The literature review which follows will introduce the different aspects and techniques used in this study.

1.1 ANTIMICROBIALS

1.1.1 Food preservation and safety

Food is a very complex substance composed mainly of carbohydrates, proteins, fats, water, minerals, vitamins, and many other components. The combination of these components is that which makes one food different from another. Moreover, each of these components are susceptible to a different form of deterioration and changes in the food cause consequences ranging from severe hazard to relatively trivial loss of quality. It is clear that all foods are perishable and some foods become unfit for human consumption more quickly than others, depending on the food type, composition, formulation, packaging and storage conditions, as well as to the length of the supply chain (Gould, 1995).

The major deteriorative reactions, which are the major targets for preservation, are well known (Table 1.1). They include some that are essentially biological, chemical, enzymatic and physical in their mode of action; although these causes are not isolated in nature.

Bacteria, insects, and light, for example, can all work simultaneously to deteriorate food in the field or in storage. Likewise, causes such as high temperature, moisture and air will affect the multiplication and activities of bacteria, as well as the chemical and enzymatic activities of the food. The most serious deterioration include those due to microorganisms, following the survival and/or growth of infectious pathogenic bacteria or the growth of toxinogenic ones (Lund *et al.*, 2000). Preservation methods ought to work through those factors that most effectively influence the growth and survival of

microorganisms and can be exploited safely in foods (Gould, 1995). Such factors include physical, chemical and microbiological and are summarized in Table 1.2.

Table 1.1 Principal quality deterioration reactions of foods

Biological	Chemical	Enzymatic	Physical
Growth or presence of toxinogenic microorganisms	Oxidative rancidity	Hydrolytic reactions catalysed by lipases, proteases, etc	Low or high temperatures
Growth or presence of infective microorganisms	Oxidative and reductive discoloration	Rancidity catalysed by lipoxygenases	Mechanical damage
Growth of spoilage microorganisms	Non-enzymic browning (Maillard reactions)	Enzymic browning catalysed by polyphenoloxidases	Excessive moisture
Presence of insects and/or rodents	Destruction of nutrients		Light
			Dehydration

Adapted from Gould (2000).

Table 1.2 Major factors affecting microbial growth and survival in foods

Intrinsic factors	Chemical and physical factors that are within the food and with which a contaminating microorganism is therefore inextricably in contact
Processing factors	Processes that are deliberately applied to a food in order to improve its preservation
Extrinsic factors	Factors which influence microorganisms in foods, but which are applied from outside the food and act during storage
Implicit factors	Factors related to the nature of the microorganisms that are present, and to their reactions with the food environment
Net effects	Effects due to the interaction of a combination of factors

Adapted from Gould (1995).

Preservation methods are of increasing importance as they delay the growth of spoilage microorganisms and guarantee the safety of the consumer by preventing or

controlling the presence of pathogens. Concerns regarding food quality and safety issues have become more important, as more food products are produced on a large industrial scale and distributed worldwide (Roller, 2003). Sometimes several months or years may elapse between food production and consumption; therefore a long-term shelf life is often necessary. Extension of the shelf life can be achieved by the use of different preservation methods (Table 1.3).

Table 1.3 Preservation methods and their antimicrobial effect.

Method	Factor influencing growth or survival
▪ Refrigeration/Freezing	Low temperature to retard growth / Reduction of water activity to prevent growth
▪ Drying, curing and conserving	Reduction in water activity to delay or prevent growth
▪ Vacuum and oxygen free packaging	Low oxygen tension inhibits strict aerobes and delays growth of facultative anaerobes
▪ Modified atmosphere packaging (MAP)	Specific inhibition of some microorganisms by carbon dioxide addition for example
▪ Addition of weak acids	Reduction of the intracellular pH of microorganisms
▪ Fermentation	Reduction of pH value *in situ* by microbial action and additional inhibition by formation of acid or alcohol and by other microbial products (e.g. bacteriocins)
▪ Emulsification	Compartmentalisation and nutrient limitation
▪ Addition of chemical preservatives	Inhibition of specific groups of microorganisms
▪ Pasteurization and appertization	High temperatures to inactivate target microorganisms
▪ Irradiation	Delivery of ionising radiation to eliminate pathogens
▪ High hydrostatic pressure (Pascalization)	Pressure-inactivation of vegetative bacteria, yeasts and moulds
▪ Pulsed electric field processing (PEF)	Short bursts of electricity for microbial inactivation

Adapted from Gould (1998).

The effectiveness of these technologies depends on a number of factors associated with the target microorganism, the food product, as well as the storage and handling environment. Thus, some technologies have limited applications. Research on food preservation technologies and their optimised application is an ongoing process and the development and use of safe antimicrobial preservatives in foods is of great interest to the industry.

Food additives are substances added to food to preserve flavour or improve its taste and appearance. Many have been in use for up to a century with a focus on food shelf life extension. Food additives are defined in European Union (EU) legislation (Article 1(2) of Directive 89/107/EEC) as "any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value, the intentional addition of which to food for a technological purpose results in it or its by-products becoming directly or indirectly a component of such foods." Additives used to prevent biological deterioration are called "antimicrobials". Antimicrobial agents are defined by the US Food and Drug Administration (FDA) 21CFR 170.3(o)(2) as "substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mould and rope inhibitors the effects listed by the National Academy of Sciences/National Research Council under 'preservatives'"

The use of antimicrobials can reduce or eliminate target microorganisms but it may also produce favourable conditions for other microorganisms (Davidson and Branen, 2005). It is recognized that this situation is less likely to develop towards substances that have more than one mode of action (Ippolito and Nigro, 2003). Hurdle technology combines existing and new preservation techniques to establish a series of preservative

factors (hurdles) that microorganisms are unable to overcome (Leistner and Gorris, 1995). These hurdles may be temperature, water activity, acidity, redox potential, preservatives, and others (Figure 1.1).

Taken from: www.habasitlink.com/link.asp?FI=1114.html

Figure 1.1 Multiple hurdle technology.

A crucial phenomenon of concern to hurdle technology is known as the homeostasis of microorganisms, which is the tendency of microorganisms to maintain a stable and balanced internal environment. Preservative factors functioning as hurdles can disturb one or more homeostasis mechanisms, thereby preventing microorganisms from multiplying and causing them to remain inactive or die. The optimum way to achieve this is to deliberately disturb several homeostasis mechanisms simultaneously (Leistner 2000; Leistner and Gould 2002). This multi-targeted approach is the essence of hurdle technology. It is more effective than single targeting and allows hurdles of lower intensity, thereby minimising impact on product quality. There is the further possibility that different hurdles in a food not only have an added effect on stability, but can act synergistically (Leistner, 1995).

1.1.2 Chemical preservatives

A preservative is a natural or synthetic chemical that is added to foods or pharmaceuticals to prevent spoilage, whether from microbial growth or undesirable chemical changes. FDA has established regulations that set out the rules on declaration of flavours, colourings, and preservatives (21 CFR §101.22). A definition of chemical preservative is contained within 21 CFR §101.22(a)(5) as "any chemical that, when added to food, tends to prevent or retard deterioration thereof, but does not include common salt, sugars, vinegars, spices, or oils extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties." In Europe, the EU Directive No 95/2/EC, defines 'preservatives' as substances which prolong the shelf life of foodstuffs by protecting them against deterioration caused by microorganisms.

There are over 80 substances which have permitted use as preservatives. Common antimicrobial preservatives include calcium propionate, sodium nitrate, sodium nitrite, sulfites (sulfur dioxide, sodium bisulfite, potassium hydrogen sulfite, etc.) and disodium EDTA. Chemical food preservatives such as benzoates, sorbates, nitrites and sulfites have been used effectively for many years, but their safety has been questioned (Knekt *et al.*, 1999). Hence restrictions apply on the levels of additives that are allowed to be added to the majority of foodstuffs. This applies not only to the levels of a preservative permitted for a particular product, but to the overall dietary intake of the preservative.

1.1.3 Natural antimicrobials

Food preservation is becoming more complex in response to changing requirements and regulations. In recent years this has included a consumer led demand for foods that are nutritionally healthier, more convenient in use (e.g. easier to store and prepare), fresher (e.g. chill-stored), more natural and therefore less heavily processed (e.g. mildly heated), less heavily preserved (e.g. less acid, salt, sugar) and less reliant on additive preservatives (e.g. sulfite, nitrite, benzoate, sorbate) than previously (Gould, 2001). Consequently, there is pressure on food manufacturers to remove or reduce the traditional chemical preservatives in their foods, and to adopt more natural alternatives with strategic combinations of technologies for the maintenance of product quality in tandem with extension of shelf life and safety. This has fuelled a search for 'bio' or naturally derived preservatives that can be safely incorporated into various food products.

A growing number of improved technologies are being researched or are in the early stages of application. Alternative strategies for microbial control and shelf life extension include the use of novel 'natural' antimicrobials, which are bioactive compounds derived from biological sources (animals, plants or microorganisms) such as bacteriocins, organic acids, plant essential oils, enzymes, etc (Table 1.4).

Ideally, these bio-preservatives have been Generally Recognized As Safe (GRAS), resulting from a long history of their association with food without associated health concerns (McLay *et al.*, 2002). Many studies have demonstrated antimicrobial activity of natural compounds against pathogenic or spoilage organisms in laboratory media. However, few of these have been translated into real food applications. Despite the wide range of potential antimicrobials, relatively few are suitable for use, in practice, in

particular food products (Roller 2003), due to having a limited spectrum of activity. They are often effective only at very high concentrations (Branen and Davidson 2004).

Table 1.4 Sources of natural antimicrobials.

Animal	Plant	Microbial
Chitosan	Essential oils	Natamycin
Lactoferrin	Organic acids	Nisin
Lactoperoxidase system	Saponins	Pediocin
Lactoglobulin	Flavonoids	Other bacteriocins
Lactolipids	Thiosulfinates	
Lysozyme	Cathechins	
Ovotransferrin	Glucosinolates	
Ovoglobulin		
Avidin		

1.1.3.1 Fatty acids and derivatives

1.1.3.1.1 Fatty acids

One group of chemicals found in nature and considered to have little or no toxicity is fatty acids and their corresponding esters. Fatty acids are carboxylic acids, regularly with a long aliphatic tail that may be either saturated or unsaturated. They are found in animal and vegetable fats and oils and have the general formula $C_nH_{2n+1}COOH$.

Fatty acids have a long historical record for antimicrobial activity; however interest in this class of natural antimicrobials has resumed in recent times. The antimicrobial effects of fatty acids have been well documented (Bergsson *et al.*, 2002; Dufour *et al.*, 2003; Glass and Johnson 2004; Kitahara *et al.*, 2006). The bactericidal

activity of lipids depends on their nature, particularly chain length, and on the bacterial strain involved. Generally, Gram-positive bacteria are lipid sensitive whereas Gram-negative bacteria are more resistant (Kabara *et al.*, 1972). Recently, however, lipid sensitive Gram-negative bacteria have been described (Petschow *et al.*, 1996; Bergsson *et al.*, 2002; Sun *et al.*, 2003). Distinct experimental conditions, such as the test medium used, pH, lipid concentrations, and probably also the bacterial strains tested, may be responsible for the observed differences in lipid sensitivity of Gram-negative bacteria (Sprong *et al.*, 2001).

The action of fatty acids results from the undissociated molecules rather than their corresponding anions. Therefore, the antimicrobial activity of fatty acids is affected by pH, since the hydrogen ion concentration determines the degree of dissociation of the acid. Antimicrobial activity has been observed at lower pH levels (4.5-5.0), in circumstances where pH alone is not lethal (Sun *et al.*, 2003). Furthermore, changes in pH affect the minimum inhibitory concentrations (MIC) of fatty acids (Oh and Marshall, 2003).

The antimicrobial effect varies with the length of the acid chain and its degree of saturation. The chain lengths of fatty acids that were found to be most effective were C12 (saturated), C16:1 (unsaturated) and C18:2 (polyunsaturated). In addition, the chain length, the degree and type of unsaturation as well as the geometric isomerism are important determinants of the biocidal activity of a fatty acid.

In studies on hydrolysis of bovine milk fat by pregastric lipase, Sun *et al.*, (2002) found that short-chain and medium-chain fatty acids are preferentially released and such hydrolysed milk fat products exert antimicrobial effects *in vitro*. The efficacy of a fatty acid as an antimicrobial is dependent on the compound being in solution but also being

sufficiently lipophilic to adsorb to the cell surface (Galbraith *et al.*, 1971). As the molecular weight of the fatty acid increases, the hydrophilic properties decrease, increasing the solubility in oil and decreasing the solubility in water. When the chain length is 12 or longer, the antimicrobial activity is greater for unsaturated fatty acids than for saturated forms (Ababouch *et al.*, 1994a; 1994b).

Fatty acids chosen for this study are described as follows:

- Lauric acid

Lauric acid (LA) or dodecanoic acid (C-12) is a medium chain saturated fatty acid with structural formula $CH_3(CH_2)_{10}COOH$ (Figure 1.2), that occurs extensively in the seed fats of plants from the *Lauraceae* or Laurel family. It is the dominant fatty acid in cinnamon oil (80–90%), coconut oil (41–56%) and palm kernel oil (41–55%). Lauric fatty acids provide up to 12% of total fat content in milk contributing as an energy source and as a natural antimicrobial.

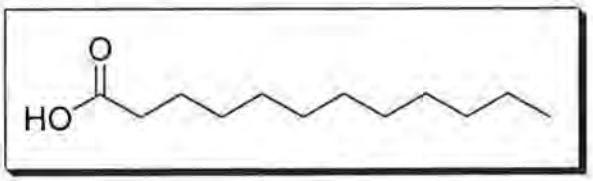


Figure 1.2 Molecular structure of lauric acid

Lauric acid, although slightly irritating to mucous membranes, has a very low toxicity and so is used in many soaps and shampoos. Sodium lauryl sulfate is the most common lauric-acid derived compound used for this purpose. Because lauric acid has a non-polar hydrocarbon tail and a polar carboxylic acid head, it can interact with polar solvents as well as fats, therefore acting as an emulsifier.

Recent studies have demonstrated useful activity of lauric acid against a number of pathogens, e.g., *Listeria monocytogenes, Salmonella enteritidis, Helicobacter pylori, Bacillus cereus* (Ababouch *et al.*, 1994a; Ababouch *et al.*, 1994b; Dawson *et al.*, 2002; Sun *et al.*, 2003; Hoffmann *et al.*, 2001), viruses (Bartolotta *et al.*, 2001) and spoilage microorganisms (Ouattara *et al.*, 1997; Ouattara *et al.*, 2000).

Lauric acid is relatively inexpensive and therefore potentially very useful and available for infection control and medical treatment in hospitals and health care environments. Lauric acid might be a potential antimicrobial material in combination with other antimicrobial agents, suitable for external application. For example, Kitahara *et al.*, (2006) found that a combination of lauric acid and gentamicin showed synergistic activity against MRSA.

- Caprylic acid

Caprylic acid (CA) or octanoic acid (C-8) is a short chain saturated fatty acid with structural formula $CH_3(CH_2)_6COOH$ (Figure 1.3). It occurs naturally in butterfat and palm and coconut oils in the form of triacylglycerols and is also found in breast milk. It is an oily liquid with a slightly unpleasant rancid taste and is minimally soluble in water. It is an intermediate in the manufacture of perfume esters and of dyes. It has also been used in organic synthesis, manufacture of medicines, rubber and latex, plastics, greases and lubricants, food additives and pharmaceuticals.

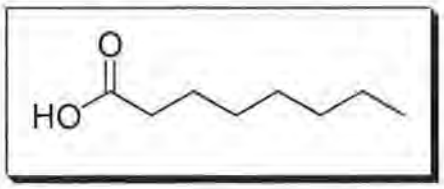


Figure 1.3 Molecular structure of caprylic acid

Galbraith *et al.*, (1971) noted that fatty acids must be in solution and remain sufficiently lipophilic to enable adsorption to bacterial cell surfaces for antibacterial activity. Caprylic acid as an 8-carbon, short-chain fatty acid, is less hydrophobic and potentially more soluble than medium-chain and long-chain fatty acids, therefore it is potentially more effective as a bactericide.

Nair *et al.*, (2005) examined the efficacy of caprylic acid against a number of pathogens, and showed that it exerts antimicrobial activity against a wide range of microorganisms including *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*. Caprylic acid and its monoglyceride, monocaprylin, can inactivate infant pathogens such as respiratory synctial virus, *Haemophilus influenza,* and group B streptococci in infant formula at 37°C (Isaacs *et al.*, 1995). Caprylic acid has been evaluated as an alternative or adjunct to antibiotics as an intramammary infusion to treat bovine mastitis (Nair *et al.*, 2005).

1.1.3.1.2 Fatty acid esters

Due to their amphiphilic nature, fatty acid esters possess emulsifying properties and exhibit inhibitory effects against some types of microorganisms. This latter property makes them promising substances for use in various aspects of the food and other industries.

Fatty acids have been esterified with monohydric alcohols resulting in inactive esters (Kabara *et al.*, 1972). Alternatively, the esterification with certain polyhydric alcohols such as glycerol or sucrose yielded active derivatives (Conley and Kabara, 1973). Monolaurin (ML) is the glycerol monoester of lauric acid (Figure 1.4), which

proved to be more active than the free acid. Dilaurin and trilaurin derivatives were less active than the monoglyceride (Kabara *et al*, 1972). Therefore, the purity of this compound is very important, as many monoglyceride preparations have a number of impurities, particularly notable quantities of di- and tri-esters.

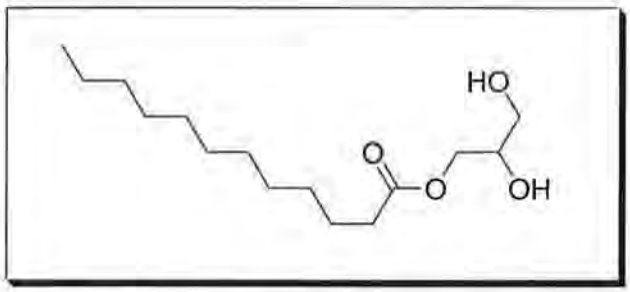


Figure 1.4 Molecular structure of lauric acid monoester.

Monolaurin is approved for use as an emulsifier in foods at levels dictated by good manufacturing practices, however, in addition to its emulsification properties; monolaurin also possesses broad spectrum antimicrobial activity against bacteria, yeasts and moulds, but generally is more active against Gram positive bacteria. The use of monolaurin in the food industry as a preservative is still limited. Nevertheless it has GRAS status by the US FDA and it is commercially available with the trade name Lauricidin® (Med-Chem Laboratories, Inc., Galena, Illinois), as a safe dietary supplement for suppression of colds and flu, although these have not been evaluated by the FDA.

Monoglycerides, especially monolaurin, have received a lot of attention due to their antimicrobial properties against a range of infectious agents including bacteria, viruses, yeast and fungi (Kabara *et al.*, 1972; Beuchat 1980; Wang and Johnson 1992; Bartolotta *et al.*, 2001; Mansour and Millierc 2001; Riháková *et al.*, 2001; Walters *et al.*,

2003). It has been demonstrated that at concentrations between 25 μg/ml and 250 μg/ml, monolaurin inhibits growth of bacteria (Branen and Davidson, 2004).

Monolaurin possesses significant antimicrobial activity against Gram-positive pathogens including *Listeria monocytogenes* (Wang and Johnson, 1992; Petrone *et al.*, 1998; Branen and Davidson, 2004; Mbandi *et al.*, 2004) and *S. aureus* (Kabara *et al.*, 1972; Schlievert *et al.*, 1992; Bergsson *et al.*, 2001; McLay *et al.*, 2002; Kitahara *et al.*, 2004; Kitahara *et al.*, 2006). Although, some Gram negative bacteria have also been found to be susceptible (Petschow *et al.*, 1996; Bergsson *et al.*, 1998; Bergsson *et al.*, 1999; Bergsson *et al.*, 2002; Sun *et al.*, 2003).

In studies with *L. monocytogenes*, Wang and Johnson (1992), observed inhibitory effects by C12:0, C18:3 and monolaurin in liquid culture media and milk. Monolaurin was bactericidal at >200 mg/ml at 4°C but was not inhibitory at 30°C. Moreover, monolaurin was less active in skim milk than in brain heart infusion broth, and inactive in whole milk. Of the fatty acids tested, lauric acid showed the highest inhibitory activity.

Wang and Johnson (1997) tested the effects of monoglycerides and their combinations on *L. monocytogenes* in foods. They observed inhibitory effects in some foods (seafood salad, pH 4.9, and beef rank slurries, pH 5.0 and 5.5), but not in several other foods of animal origin. When 200 μg/L monolaurin was combined with 0.5% lactic acid, significant inhibitory effects were observed for *Listeria monocytogenes* (Oh and Marshall, 1995).

Monolaurin increased the sensitivity of *Bacillus cereus* spores to thermal inactivation (Chaibi *et al.*, 1998), and when combined with monocaprin inhibited growth of *L. monocytogenes* (Wang and Johnson, 1997).

1.1.3.1.3 Fatty acid ether derivatives

The alkylglycerol ether (dodecylglycerol) (Figure 1.5) has been synthesised by Ved *et al.*, (1984) who showed that depending upon the incubation conditions, this compound can display more than 2-fold higher antibacterial activity than monolaurin, the acylglycerol ester. As with the monoglyceride, alkylglycerol ether was more active against Gram positive than Gram negative bacteria and the antibacterial activity was higher at a chain length of 12 carbons (Ved *et al.*, 1984).

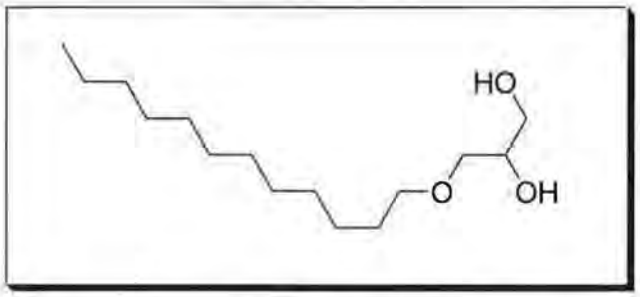


Figure 1.5 Dodecylglycerol

1.1.3.1.4 Carbohydrate fatty acid derivatives

Carbohydrate fatty acid (CFA) esters are another class of fatty acid derivatives which have wide applications in the food and health-care industry (Watanabe, 1999). They are used as emulsifiers, non-ionic surfactants, bleaching boosters and food additives. Another attractive feature of carbohydrate fatty acid derivatives is the potential to modify their properties by controlling the degree of substitution of the carbohydrate, by varying the nature of the fatty acid and also the sugar itself. Sugar fatty acid esters with degrees of substitution of 1 to 3 are non-ionic, digestible, absorbable and biodegradable detergents of low toxicity. Fully esterified carbohydrate fatty acid

derivatives are lipophilic, indigestible and unabsorbable molecules and have been used as fat substitutes such as Olestra® (Procter & Gamble).

Sugar esters are used in Japan as antibacterial agents in canned drinks (Suwa *et al.*, 1988, Moriyama *et al.*, 1996, Mitsubishi-Kagaku Foods Corporation, 2009), although in Europe and USA their use in the food industry as a preservative is still limited. Sucrose esters of fatty acids (E 473) are food additives permitted in the European Union for use as emulsifiers and stabilisers for oil/water emulsions in several processed foods by Directive 95/2/EC on food additives other than colours and sweeteners. The most common carbohydrate fatty acid ester utilised to date is sucrose ester, which is produced at about 4000 ton/year (Ferrer *et al.*, 2005). The use of carbohydrate esters is continually increasing as they are completely biodegradable; they are not harmful to the environment and they are non-toxic (Janssen *et al.*, 1991).

Carbohydrate fatty acid esters have been synthesised chemically and enzymatically by interesterification, transesterification and direct esterification. Enzymatic methods can allow regioselectivity; nevertheless, they do not allow any flexibility in the regiomeric outcome. To date, chemical methods have not given rise to regioselectivity, and have also produced mixtures of mono, di- and tri-esters. Recent work in the area of carbohydrate fatty acid esters has been focused on establishing an effective regioselective enzyme catalysed synthesis of sugar derivatives as surfactants for industrial applications (Fregapane *et al.*, 1991; Tsuzuki *et al.*, 1999; Ferrer *et al.*, 2000; Devulapalle, 2004).

However, little work has been done on investigating the role of the carbohydrate in antimicrobial activity. Conley and Kabara, 1973, examined the antimicrobial activity of a range of sucrose esters and concluded that they had greater efficacy than the

corresponding free fatty acid. Diesters of sucrose have been found to be more active than the monoesters, which is in contrast with data for the glycerides (Kato and Shibasaki, 1975; Beuchat, 1980).

1.1.3.2 Essential oils

Essential oils (EOs) are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). Distillation is the most popular, and cost effective method in use today for producing essential oils. Other processes include expression, solvent extraction, fermentation or enfleurage. An estimated 3,000 EOs are known, of which about 300 are commercially important for the food (as flavourings), health-care (fragrances) and pharmaceutical (for their functional properties) industries (Burt, 2004).

The antimicrobial activity of EOs have long been recognised and demonstrated against a number of microorganisms (Smith-Palmer *et al.*, 1998; Hammer *et al.*, 1999; Dorman and Deans, 2000; Holley and Patel, 2005; Moreira *et al.*, 2005; Oussalah *et al.*, 2007; Gutierrez *et al.*, 2008). A small number of food preservatives containing EOs are already commercially available. "DMC Base Natural" is a food preservative produced by DOMCA S.A. (Alhendín, Granada, Spain) and comprises 50% essential oils from rosemary, sage and citrus and 50% glycerol (Mendoza-Yepes *et al.*, 1997). "Protecta One" and "Protecta Two" are blended herb extracts produced by Bavaria Corp. (Apopka, FL, USA), consisting of one or more EOs dispersed in solutions of sodium citrate and sodium chloride, respectively (Cutter, 2000).

EOs can comprise more than sixty individual components. Major components can constitute up to 85% of the EO, whereas other components are present only as a trace

(Senatore, 1996). The phenolic components are chiefly responsible for the antibacterial properties of EOs (Cosentino *et al.*, 1999). The major components of a number of EOs with antibacterial properties are presented in (Table 1.5).

Table 1.5 Major components of selected EOs that exhibit antibacterial properties.

Common name	Latin name of plant source	Major components (Approx. %composition)
Cilantro	*Coriandrum sativum* (immature leaves)	Linalool 26% E-2-decanal 20%
Coriander	*Coriandrum sativum* (seeds)	Linalool 70%
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde 65%
Oregano	*Origanum vulgare*	Carvacrol 0-80% Thymol 0-64% γ-Terpinene 2 –52% p-Cymene Trace-52%
Rosemary	*Rosmarinus officinalis*	α-pinene 2 –25% Bornyl acetate 0 –17% Camphor 2 –14% 1,8-cineole 3 –89%
Sage	*Salvia officinalis L.*	Camphor 6 –15% α-Pinene 4 – 5% β-pinene 2 –10% 1,8-cineole 6 –14% α-tujone 20–42%
Clove (bud)	*Syzygium aromaticum*	Eugenol 75–85% Eugenyl acetate 8 –15%
Thyme	*Thymus vulgaris*	Thymol 10–64% Carvacrol 2 – 11% γ-Terpinene 2 –31% p-Cymene 10–56%

Adapted from Burt, 2004.

Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) are amongst the most active EOs. Several essential oil components have been reported as efficient antibacterial or antifungal agents, such as linalool (Knobloch *et al.*, 1989), 1,8-cineole (Sökmen *et al.*, 2004), α-terpineol, terpinen-4-ol, α-pinene, β-pinene, β-caryophyllene, α-phellandrene, *p*-cymene (Dorman and Deans, 2000).

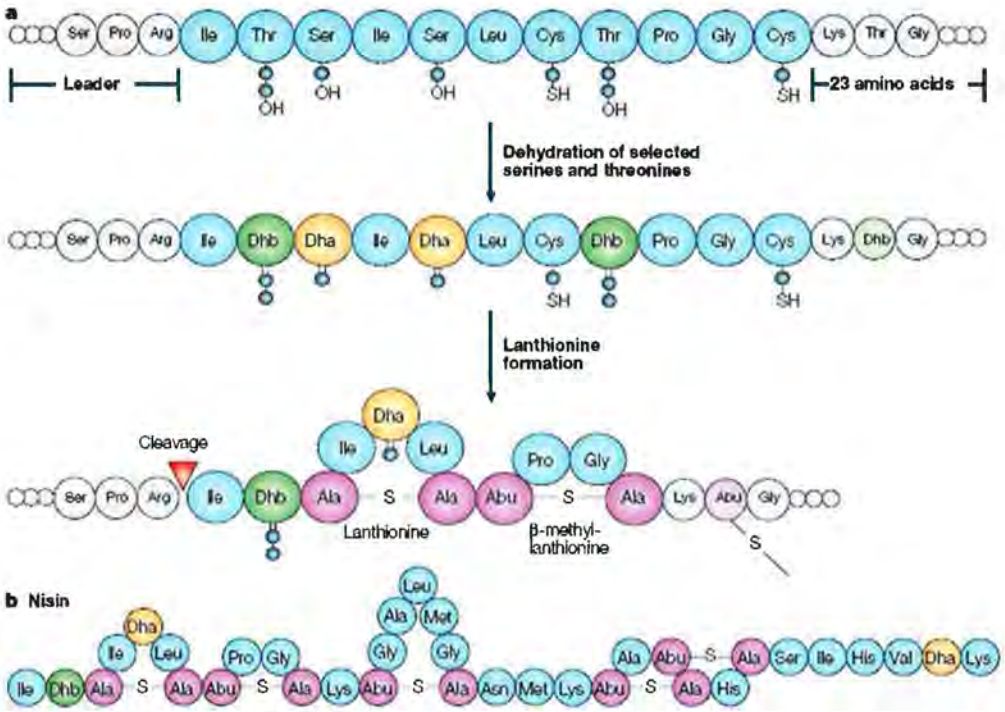
As potential antimicrobial agents, the natural occurrence of EOs as part of the human diet and their biodegradability, suggest low toxic residue problems (Nedorostova *et al.*, 2009). A number of EO components have been registered by the European Commission for use as flavourings in foodstuffs (Commission Decision of 23 January, 2002). The flavourings registered are considered to present no risk to the health of the consumer. The EU registered flavourings also appear on the "Everything Added to Food in the US" list, which means that the US FDA has classified the substances as Generally Recognised As Safe (GRAS) or as approved food additives.

1.1.3.3 Bacteriocins

Bacteriocins produced by lactic acid bacteria (LAB) are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes that have a bactericidal or bacteriostatic effect on other species (Nes *et al.*, 1996). Bacteriocins from LAB have been largely studied with the perspective of food protection against pathogenic and spoilage bacteria (Cleveland *et al.*, 2001; O'Sullivan *et al.*, 2002). Although bacteriocins are produced by many Gram-positive and Gram-negative species, those produced by the LAB are of particular interest to the food industry (Nettles and Barefoot, 1993), since these bacteria are natural food isolates and have generally been regarded as safe (GRAS status) (Deegan *et al.*, 2006).

Bacteriocins are a heterogeneous group of peptides and proteins and as many as five main classes of LAB bacteriocins have been mooted (Klaenhammer, 1993; Nes *et al.*, 1996; Kemperman *et al.*, 2003). Cotter *et al.* (2005) proposed a revised classification scheme which divides the bacteriocins into two distinct categories: the lanthionine-containing Lantibiotics (class I) and the non-lanthionine-containing bacteriocins (class II), while moving the large, heat-labile murein hydrolases (formerly class III bacteriocins) to a separate designation called 'bacteriolysins'.

Nisin is a polycyclic peptide antibacterial with 34 amino acid residues, and a member of the group of related polypeptides termed lantibiotics. It is a heatstable peptide extensively modified after translation, resulting in the formation of characteristic thioether amino acids lanthionine (Lan) and methyllanthionine (MeLan) (Jack and Sahl, 1995). These are derived from a two-step process: firstly, gene-encoded serine and threonine can be subject to enzymatic dehydration to give rise to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Then, thiol groups from neighbouring cysteines attack the double bond of Dha or Dhb yielding either Lan or MeLan, respectively (Figure 1.6). This condensation between two neighbouring residues results in the formation of covalently closed rings within the formerly linear peptide, conferring both structure and functionality.



Adapted from Cotter *et al.*, 2005

Figure 1.6 Lanthionine synthesis (a) and nisin structure (b).

Nisin forms pores that disrupt the proton motive force and the pH equilibrium causing leakage of ions and hydrolysis of ATP resulting in cell death (de Arauz *et al.*, 2009). Lipid II, a docking molecule or target, is involved in several cases in the mechanisms of action of nisin (Brotz *et al.*, 1998). The binding of nisin to lipid II facilitates a dual mechanism of action involving pore formation and preventing peptidoglycan synthesis (Breukink *et al.* 1999; Wiedemann *et al.*, 2001).

Nisin remains the most commercially important bacteriocin, although other bacteriocins have been characterized and developed for possible approval and use (Cleveland *et al.*, 2001). Nisin A and pediocin PA-1, which are the only bacteriocins approved for utilization as a preservative or ingredient, are produced by *Lactococcus lactis* and *Pediococcus acidilactici*, respectively. Nisin is commercially available as

Nisaplin™ (Danisco, Copenhagen, Denmark), while pediocin PA-1 is marketed as ALTA™ 2341 (Kerry Bioscience, Carrigaline, Co. Cork, Ireland).

A major problem with bacteriocins application is related to the *in situ* antimicrobial efficacy, which can be negatively influenced by various factors, such as binding to food components, inactivation by proteases, changes in solubility and charge, or changes in the cell envelope of the target bacteria (Jung *et al.*, 1992; Aesen *et al.*, 2003). The chemical composition and the physical conditions of food can have a significant influence on the activity of the bacteriocin. Nisin, for example, is 228 times more soluble at pH 2 than at pH 8 (Liu and Hansen, 1990). Nisin has been shown to be effective in a number of food systems, including dairy products, canned foods and processed cheese inhibiting the growth of a wide range of bacteria, (Cutter and Siragusa, 1995; Ferreira and Lund, 1996; Davies *et al.*, 1999).

Although some LAB bacteriocins have been found to be active against Gram-negative bacteria, they are generally effective against closely related Gram-positive species. The addition of bacteriocins along with other natural preservatives might increase their efficacy within food matrices against some Gram-negative spoilage bacteria that are known to show consistently high resistance to antimicrobials, such as *Pseudomonas* spp. (Hammer *et al.*, 1999; Holley and Patel, 2005).

1.1.4 Antimicrobial agents and resistance

Antimicrobials are natural or synthetic drugs which inhibit or kill bacteria. This capability makes them unique for the control of deadly infectious diseases caused by a large variety of pathogenic bacteria. Antibiotics, also referred as chemotherapeutic agents or antimicrobial drugs, are used predominantly for the treatment of bacterial

infections in humans and animals; whereas biocides are employed for their antiseptic, disinfectant and/or preservative properties.

Today, more than 15 different classes of antimicrobial drugs are known. They differ in chemical structure and mechanism of action. Specific antimicrobial drugs can be necessary for the treatment of specific pathogens. Following their 20th century triumph in human medicine, antimicrobials have also been used increasingly for the treatment of bacterial disease in animals, fish and plants. In addition, they became an important element of intense animal husbandry because of their observed growth-enhancing effect, when added in sub-therapeutic doses to animal feed.

The persistent emergence of antibiotic-resistant strains of pathogens is making successful empirical therapy much more difficult to achieve, as antibiotic choices are often severely restricted (Gould, 2009). The increasingly rapid pace at which microbes are able to develop or acquire new drug resistance profiles outpaces the rate at which the pharmaceutical industry develops, screens and distributes new antimicrobial agents (Cloutier, 1995). Thus there is a need for alternative approaches, some products used in the past seem to be making a return, and other alternatives are being investigated.

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are increasing in both hospital and community settings. *S. aureus* is one of the most common causes of bloodstream infection (or bacteremia). MRSA is a global concern, in Europe, the highest rates of MRSA bacteraemia in 2007 were observed in Malta (52.4%), Portugal (48.4%), Cyprus (48.2%), Greece (48%), Ireland (38.1%), Croatia (37.6%) and UK (35.6%). The lowest rates (all <2%) were observed in Scandinavian countries and the Netherlands (EARSS, 2007). In Ireland, the annual trend decreased from approximately 42% between 2002 and 2006 to 38.5% in 2007 (HPSC, 2007).

Consequently, there has been considerable interest in discovering and developing new antistaphylococcal agents for potential therapeutic application.

Antimicrobial resistance continues to be a growing problem including other pathogens such as enterococci and *E. coli* (Schmidt *et al.*, 1998). Therefore, measures to promote prudent antibiotic use in both hospital and community settings are required to reduce the burden of antimicrobial resistance.

Acquisition of antibiotic resistance genes is the predominant factor in the emergence, evolution and spread of antibiotic resistant bacteria (Hall and Collis, 2001). Antibiotic-resistant bacteria are increasingly seen to be just as virulent as their sensitive counterparts, and their genetic adaptability gives bacteria a huge advantage over mankind (Lindsay and Holden, 2006). This situation arises as a result of the excessive and often inappropriate use of antibiotics in human and animal health care for the treatment and prevention of bacterial infections (Rao, 1998; Lohner and Staudegger, 2001).

Development of antimicrobial resistant foodborne bacterial pathogens can potentially compromise human drug treatments. It is now collectively acknowledged that the use of antimicrobials in both animals and humans can select for resistant bacterial populations (Threlfall *et al.*, 2000; White *et al.*, 2002). There is concern that antimicrobial resistant bacteria in animals may transfer to humans through the food chain (Khachatourians, 1998; Overarching AMR Report UK, 2004). While the links between animal agriculture and human disease are complicated and in need of additional study, evidence is strong enough for scientists and public health organizations to call for reduced use of antibiotics in agriculture (Goldman, 2004). Intense animal production in the agricultural industry sometimes involves giving livestock animals' large quantities of

antibiotics to promote growth and prevent infection. These uses promote the selection of antibiotic resistance in bacterial populations.

It is now more than a decade since the European Union started the withdrawal of selected growth-promoting antibiotics (Phillips, 2007). By 2006, the use of antimicrobial agents for growth promotion was no longer approved in the EU (Regulation 1831/2003/EC on additives for use in animal nutrition); however, this use of antimicrobial agents for food animals continues in North America and Australia.

1.2 MICROORGANISMS INVESTIGATED

Despite improved understanding of infection processes, better methods for controlling microorganisms and much stricter regulation of food production, foodborne diseases are still a major cause of morbidity and mortality worldwide (Roller, 2003).

Among the aims of this work is to assess the efficacy of novel antimicrobial compounds for possible applications in fields ranging from the food industry to the healthcare industry. Thus, a number of gram positive and gram negative bacteria of concern for these industries were investigated.

1.2.1 Listeria

Listeria species are Gram positive non-sporeformer bacilli which are ubiquitous in the environment and found world-wide. *Listeria monocytogenes* is the only important human pathogen among the six species currently recognized within the genus, although other species have occasionally been associated with human illness. They are not highly

pathogenic to healthy adults, however, pregnant women, neonates, the elderly and the immuno-compromised are at particular risk and disease in these groups can be severe.

L. monocytogenes causes the disease listeriosis, which has several manifestations such as meningitis, septicaemia, encephalitis, and cervical infections in pregnant women, which may result in spontaneous abortion (2nd/3rd trimester) or stillbirth. The infective dose of *L. monocytogenes* is unknown but is believed to vary with the strain and susceptibility of the victim. From cases contracted through raw or supposedly pasteurized milk, it is safe to assume that in susceptible persons, fewer than 1,000 total organisms may cause disease (FDA, 2009). Due to the long incubation time, the tracing and identification of the pathogen in the contaminated food can be very difficult.

Listeria monocytogenes is currently a worldwide concern affecting numerous countries. In the EU, in addition to the economic consequences and threats associated with outbreaks, listeriosis remains of great public health concern, as it has one of the highest case fatality rates of all the foodborne infections: 20-30% (De Valk *et al.*, 2005). In 2007, 1,554 confirmed cases of human listeriosis were reported in 24 EU Member States and was the fifth most common zoonotic infection in Europe, after *Campylobacter, Salmonella, Yersinia*, and VTEC infections (EFSA, 2009). Despite the food controls carried out in EU Member States, the incidences of listeriosis, outbreaks and contaminated product recalls continue to occur. *L. monocytogenes* is widely distributed in nature, can adapt to survive and grow in a wide range of environmental conditions, being frequently found in a large variety of raw and processed foods, as well as in processing plants (Rocourt and Cossart, 1997). Foods most likely to be contaminated are unpasteurized cheeses, cold cuts of meat, pâtés and smoked fish, or ready meals which have been pre-cooked and then chilled for some time before

consumption. *L. monocytogenes* has emerged as one of the most important food pathogens in ready-to-eat processed meals and dairy foods (FSAI, 2005) due to its survival and growth at refrigeration temperatures (2–4 °C) making the control of this foodborne pathogen difficult, since refrigeration is one of the most common ways to increase the shelf life of ready-to-eat and minimally processed foods.

1.2.2 Staphylococcus aureus

S. aureus is a Gram-positive coccus. When viewed microscopically, *S. aureus* appears in clusters, like bunches of grapes. *S. aureus* belongs to the genus Staphylococcus, together with other species, such as *S. intermedius*, *S. hyicus* and *S. epidermidis*. *S. aureus* can be distinguished from *S. epidermidis* by the production of the enzymes coagulase and thermonuclease.

The main reservoirs of *S. aureus* are humans and animals. Healthy people carry the organism in their nose and throat (50 %), on their hands (5-30 %), and in wounds. *S. aureus* can also colonize food contact surfaces, and it can become a persistent organism in slaughterhouses. *S. aureus* can contaminate foods through contact with contaminated hands, materials and surfaces, but also via the air (coughing). Growing in food, some strains can produce toxins which cause acute gastro-intestinal diseases if ingested. The enterotoxin produced by *S. aureus* is a heat-stable protein, which survives heating at 100 °C for 30 – 700 minutes. The organism can grow both with and without oxygen (facultatively anaerobic). It is characteristic that staphylococci can grow at low water activity (approx. 0.86), corresponding with a salt content of about 14 %. *S. aureus* is extremely heat sensitive and is readily inactivated at temperatures > 46 °C.

Illness is caused by the consumption of a foodstuff contaminated with the toxin which *S. aureus* may have produced. In order to produce detectable levels of toxin, the number of organisms must be over 10^{5-6} per gram of product. The time between ingestion of the toxin and display of symptoms is only two to five hours and depends on the amount and type of food and the state of health of the person. The main symptoms are nausea, vomiting, abdominal cramps and exhaustion. Patients usually recover within two days.

Outbreaks and sporadic cases of staphylococcal food poisoning have been linked with foods such as cheese, salami, bakery products, pasta, canned meat, canned fish and canned vegetable products. What these products have in common is that they require frequent manual handling during preparation and that they are often kept at room temperature for some time. Most food intoxications caused by *S. aureus* are the result of poor hygiene practices in household and industrial kitchens. Staphylococcal food poisoning has been a notifiable disease in Ireland since 1st January 2004 (S.I No.707, 2003).

1.2.2.1 Methicillin-resistant *Staphylococcus aureus*

Methicillin-resistant *S. aureus* (MRSA) infection first emerged in early 1961 and has become increasingly prevalent, with serious infections becoming more widespread during the past 20-25 years (Kitahara *et al.*, 2006).

S. 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, 1981; 1984; 1986). Expression of mecA yields PBP2a, a

penicillin binding protein with reduced affinity for β-lactam antibiotic binding. Penicillin binding proteins are necessary for correct synthesis of the bacterial cell wall, and when they are blocked by penicillin, the cell wall is incorrectly formed, and the cell is liable to lyse. PBP2a allows the bacterium to synthesise cell wall normally in the presence of methicillin (Pinho *et al.*, 2001).

MRSA has recently become a major cause of hospital-acquired infections and is being recognized with increasing frequency in community acquired infections. *S. aureus* is one of the commonest causes of bloodstream infection (or bacteremia).

MRSA has caused serious problems in the empirical use of all the major classes of antibiotics in common use, for example, the cephalosporins, penicillins, carbapenems, quinolones and aminoglycosides. Moreover, MRSA has not replaced methicillin-susceptible *S. aureus* (MSSA), but is an additional burden of infection, often doubling or trebling the number of clinical staphylococcal infections, whether in the hospital or the community (Gould, 2005). Outcomes are worse for MRSA infection than for MSSA, for complex reasons including inappropriate empirical treatment (Cosgrove *et al.*, 2006). Possibly the major cause of this inferior outcome in MRSA infection is the reliance on glycopeptides, which have long been known to be suboptimal for MSSA infections (Gould, 2007). Indeed vancomycin, launched in 1958 on the evidence of successful treatment in just a handful of patients, was soon dropped from routine clinical use when the semisynthetic penicillins were introduced in the 1960s. Unfortunately, we still await the development of equivalent drugs for the treatment of MRSA. All new agents and those likely to be marketed in the foreseeable future, have significant drawbacks.

1.2.3 Escherichia coli

Escherichia coli are Gram negative rod-shaped bacteria that normally inhabit the intestines of humans and warm-blooded animals. Most strains are known to be harmless, but several of them can cause mild to serious disease. Verotoxigenic *E. coli* (VTEC), and in particular *E. coli* O157:H7, can cause severe diarrhoea and in some cases lead to serious complications, even death.

E. coli is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products and raw milk. Meat can become contaminated during slaughter, and organisms can be thoroughly mixed into beef when it is minced. Eating meat (especially minced beef) that has not been cooked sufficiently to kill *E. coli* O157:H7 has caused infection (Adams and Moss, 2000). *E. coli* O157:H7 has also been found in other animals such as sheep, pigs, goats, poultry, and deer. Other risk factors include drinking or swimming in contaminated water, handling animal faeces, and eating fruit and vegetables fertilized or irrigated with animal manure. Poor personal hygiene and unsafe food preparation practices also increase the risk of spreading O157:H7 infection (HPSC, 2007).

E. coli O157:H7 has been detected throughout the world, but most outbreaks have been documented in Canada, United Kingdom and USA (Adams and Moss, 2000). The reported incidence of VTEC disease in Ireland has risen from 2.4 per 100,000 in 2003 to 3.7 per 100,000 in 2006 (Garvey *et al.*, 2008; EFSA, 2009). People from any age group are susceptible to infection with *E. coli* O157:H7. However, children, the elderly and immuno-compromised persons are more likely to become infected and develop complications.

The symptoms of O157:H7 infection includes severe diarrhoea and abdominal cramps, often developing into bloody diarrhoea. Symptoms usually occur three to four days after exposure. Some people infected by *E. coli* O157:H7 may have no symptoms at all or only mild diarrhoea. Complications include the haemolytic uremic syndrome (HUS) which results in destruction of red blood cells and kidney failure. HUS can also affect the nervous system causing seizures and eventually coma. Treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents, including fluoroquinolones (Sabate *et al.*, 2008).

1.2.4 Salmonella

Salmonella is a genus of rod-shaped Gram-negative enterobacteria. *Salmonella enterica* are involved in causing diseases of the intestines. The three main serovars of *Salmonella enterica* are Typhimurium (until recently, the most common cause of food poisoning by *Salmonella* species), Enteritidis (common cause of food poisoning), and Typhi (causative agent of typhoid fever). In humans *S.* Typhimurium does not cause as severe disease as *S.* Typhi, and is not normally fatal. The disease is characterized by diarrhoea, abdominal cramps, vomiting and nausea, and generally lasts up to 7 days. *Salmonella* infections can often be fatal for immuno-compromised or elderly people if they are not treated with antibiotics. *Salmonella* occurs in raw poultry, eggs, beef, and sometimes on unwashed fruit and vegetables.

Salmonellosis remained the second most commonly reported zoonotic disease in the EU. In 2007, 456 cases of salmonellosis were notified in Ireland (HPSC, 2007), with a national crude incidence rate for 2007 of 10.7 per 100,000.

1.2.5 Pseudomonas

Pseudomonas is a genus of gram negative, aerobic, non spore forming bacilli, motile by means of one or more polar flagella. *Pseudomonas* species normally inhabit soil, water, and vegetation and can be isolated from the skin, throat, and stool of healthy persons. As a result of their metabolic diversity, ability to grow at low temperatures and ubiquitous nature, many *Pseudomonas* can cause food spoilage.

Pseudomonas aeruginosa is increasingly recognized as an emerging opportunistic pathogen of humans. They often colonize hospital food, sinks, taps, mops, and respiratory equipment causing urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteraemia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immuno-suppressed.

Pseudomonas fluorescens has multiple flagella. The name 'fluorescens' is due to the fact that they secrete a soluble fluorescent pigment called pyoverdin (formerly called fluorescein), that diffuses into the medium and is fluorescent under ultraviolet light. Pyoverdin is a siderophore, a bacterial product synthesised in response to iron limitation (Cox and Adams, 1985), functioning as a powerful iron chelator, solubilizing and transporting iron through the bacterial membrane via specific receptor proteins at the level of the outer membrane (Schons *et al.*, 2005).

P. fluorescens has an extremely versatile metabolism, and can be found in the soil and in water. It is an obligate aerobe but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration. Optimal temperatures for growth are 25-30 °C. Heat stable lipases and proteases are produced by *P. fluorescens* and other similar pseudomonas. These enzymes cause milk to spoil, by

causing bitterness, casein breakdown, and ropiness due to production of slime and coagulation of proteins.

1.2.6 Enterobacter

Enterobacter bacteria are gram negative, motile, rod shaped bacteria of the family *Enterobacteriaceae*. They are facultative anaerobes and possess peritrichous flagella. *Enterobacter aerogenes* is considered as one of the main species of this genus. *E. aerogenes* is generally found in the human intestinal tract and does not generally cause disease in healthy individuals. *E. aerogenes* has been isolated from soil, water, vegetables, meats, plants, dairy products, and cosmetics. Several strains are pathogenic and cause opportunistic infections in immuno-compromised people.

1.2.7 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are a group of related Gram-positive, non-spore-forming strictly fermentative bacteria producing lactic acid as the major end product of carbohydrate fermentation. The genera that comprise the LAB are at its core *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*. These microbes are broadly used in the production of fermented food products, such as yogurt (*Streptococcus* spp. and *Lactobacillus* spp.), cheeses (*Lactococcus* spp.), sauerkraut (*Leuconostoc* spp.) and sausage.

Though lactobacilli do not negatively affect human health, they represent problems and losses for food manufacturers, because they alter the sensorial properties of some food products. LAB are characterized by an increased tolerance to a lower pH range,

therefore during storage favoured by anaerobic conditions, psychrotrophic LAB usually become the dominant bacterial group or occur in very high numbers. LAB may spoil a product if it is not manufactured and stored correctly.

Previous studies have shown LAB to be relevant in the spoilage flora of chill stored vacuum-packaged cold-smoked fish products (Lyhs *et al.*, 1999; Gonzalez-Rodriguez *et al.*, 2002; Joffraud *et al.*, 2006) and salted and fermented ready-to-eat fish products (Lyhs and Bjorkroth, 2008). *Lb. plantarum, Lb. brevis* and *B. coagulans* are bacteria of considerable concern during the processing of acidic and acidified foods, as they are amongst the most resistant microorganism to the thermal treatments usually used by producers of fruit juices (Edwards *et al.*, 1998). Others lactobacilli such as *Lb. casei, Lb. plantarum* and *Lb. brevis* can also grow in and cause spoilage of delicacy products, especially mayonnaise-based salads (Delaquis *et al.*, 1997). The important role played by LAB in spoilage of packed, cold stored meat products has been widely reported (Chenoll *et al.*, 2007) with the species *Lb. curvatus, Lb. sakei, Lb. plantarum* and *Leuconostoc* spp. being among those most frequently identified.

1.3 EVALUATION OF ANTIMICROBIAL ACTIVITY

Currently there is no standard reproducible method for assessing the antimicrobial activity of natural antimicrobials, including plant extracts and their active components (King *et al.*, 2008). Inhibition of microbial growth can be detected using a variety of methods, including direct microscopic counts, absorbance, assays that incorporate a colorimetric and fluorometric growth indicator, bioluminescence assays, turbidity, dry weight, and zones of inhibition.

An alternative measure of susceptibility is to determine the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of an antimicrobial. A series of broths are mixed with serially diluted antimicrobial solutions and a standard inoculum is applied. After an overnight incubation, the MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. The more resistant an organism is, then the higher will be the MIC. The MBC is not as commonly seen as the Minimum inhibitory concentration (MIC). It can be determined from broth dilution MIC tests by subculturing the broths to agar media without antimicrobial. The MBC is the first dilution at which no growth is observed. Antimicrobials are usually regarded as bactericidal if the MBC is no more than four times the MIC. With static agents, the MIC is much lower than the MBC.

The most commonly used methods to determine the MIC of an antimicrobial agent are briefly described below.

- **Disk-diffusion method**

Diffusion methods have been widely used to investigate the antibacterial activity of potential antimicrobial compounds. The organism is inoculated evenly onto an agar plate to obtain a confluent lawn of growth. Disks impregnated with antimicrobial agents are then placed onto the agar. After a suitable incubation period, the plate is examined for zones of inhibited growth around the disk. The size of the zone of inhibition is inversely proportional to the MIC of the antimicrobial for the organism. This method is an indirect measure of the susceptibility based on MIC zone size correlation. Depending on the zone's size, the organism is reported to be susceptible, intermediate, or resistant to the antimicrobial agent.

The test however, must be performed under controlled and standardized conditions to provide accurate results. For example, the appropriate culture media to use, inoculum size and concentration, disk potency and its proper storage. These assays are qualitative; a disadvantage is that interpretive charts are needed and they vary with the organism being tested. The disk diffusion method can lead to problems of interpretation such as growth in the inhibition zone and subjectivity associated with visual assessments (Deighton and Balkau 1990). Diffusion methods are not the best choice for testing non polar or other samples that do not diffuse in the media well (Cos *et al.*, 2006). The hydrophobic nature of most essential oils and phenolic compounds present in plant extracts prevents the uniform diffusion of these substances through the agar medium and will therefore impact the evaluation of their antibacterial capability. In general, the relative antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility and diffusion characteristics in agar (Cos *et al.*, 2006).

- **Agar-well diffusion method**

It is another type of diffusion technique where the antimicrobial agent is placed into wells bored into an inoculated agar plate; this diffuses into the agar around the well, and is assayed for an ability to produce an inhibition zone. The hole-punch method is the only suitable diffusion technique for aqueous extracts, because interference by particulate matter is much less than with other types of reservoirs.

- **Agar dilution method**

This is one of the most commonly employed methods. Different concentrations of antimicrobial agents are incorporated onto agar plates. These are spot inoculated with a multipoint inoculator on the surface of the agar with an inoculum equivalent to 4 to 5 log CFU per spot applied. Plates are incubated at 35°C overnight and read by observing the lowest concentration that inhibits visible bacterial growth. This concentration is reported as the MIC. This method provides reliable results, but it is very time-consuming and uses a great quantity of material. In general, dilution methods are appropriate for assaying polar and non-polar extracts or compounds for determination of MIC and MBC-values.

- **Macro broth dilution method (Tube dilution test)**

This method uses tubes with broth containing a test level of antimicrobial agent, into which an inoculum of microbes is added. The end result of the test is the minimum concentration of antimicrobial which gave a clear solution, i.e., no visual growth. Although this test is fairly precise, it is laborious as serial dilutions of the antibiotic must be made and only one isolate can be tested in each series of dilutions.

- **Micro broth dilution method**

The broth microdilution method is a fast and low-cost method for MIC determination, is a reference method from the National Committee for Clinical Laboratory Standards (NCCLS) and is the method chosen for this study.

Microtiter plate-based assays have been developed for a wide range of applications including monitoring of bioactive compound production in fermentation samples (Casey

et al., 2004), determination of antimicrobial susceptibility patterns of microorganisms (Ruzicka *et al.*, 2003), and to quantify *in vitro* the synergistic activity of "natural" antimicrobials (Dufour *et al.*, 2003). The broth microdilution method appears to be an easy and reliable method for determination of the MICs of antibiotics for *C. jejuni* and *C. coli*, and it may offer an interesting alternative to MIC determination by the agar dilution technique or the E test (Luber *et al.*, 2003). Steward *et al.*, 1999 compared the automated susceptibility instruments used by most clinical laboratories, MicroScan and Vitek, and found that they did not perform well compared to broth microdilution because of the high level of minor errors, although many of the errors were within one dilution of the reference value.

1.4 ANTIMICROBIAL MODES OF ACTION

The basic mechanisms of action of antibiotics are generally well understood. Most antibiotics in clinical usage are directed against bacterial cell wall synthesis, protein synthesis, or nucleic acid synthesis, which are unique in some ways to bacteria. For example, antibiotics that inhibit peptidoglycan synthesis in the cell wall do not affect mammalian cells as they have neither a cell wall nor peptidoglycan. Other antibiotics target bacterial protein synthesis because bacterial ribosomes (termed 70S ribosomes) are different from the ribosomes (80S) of humans and other eukaryotic organisms. Antibiotics such as the fluoroquinolones inhibit prokaryotic (not eukaryotic) DNA replication, and rifamycins inhibit bacterial (not eukaryotic) DNA transcription. In addition, some antibiotics, like polymyxins, interfere with membrane integrity. However, membrane inhibition or disruption is not always successful due to similarities

in phospholipids in eubacterial and eukaryotic membranes, therefore these types of compounds are not recommended to be used systemically.

By contrast, the mechanisms of antibacterial action of biocides, which comprise preservatives, antiseptics and disinfectants, are poorly understood (Russell, 2003). Biocides are known to interact with bacterial cell walls or envelopes, produce changes in cytoplasmic membrane integrity (cationic agents), dissipate the proton-motive force (organic acids and esters), inhibit membrane enzymes (thiol interactors), act as alkylating agents (ethylene oxide), cross-linking agents (aldehydes) and intercalating agents (acridines), or otherwise interact with identifiable chemical groups in the cell (Denyer and Stewart, 1998).

1.4.1 Antimicrobial modes of action of fatty acids and derivatives

Fatty acids and their esters have several modes of action that are non-specific. The mechanism of monolaurin inhibitory action is not completely known, but it is thought to affect the cell membrane. The modes of action relevant to this study are further discussed below.

- **Membrane inhibition or disruption**

The integrity of the cytoplasmic and outer membranes is vital to bacteria, and compounds that disorganize the membranes rapidly kill the cells. However, due to the similarities in phospholipids in eubacterial and eukaryotic membranes, this action is rarely specific enough to permit these compounds to be used systemically and is therefore usually limited to topical usage.

In most cases, antimicrobial agents that work in this manner create pores in the membrane that cause the leakage of cytoplasmic contents. In addition, they may also interfere with the functions of enzymes in the membrane.

The inhibitory action of monolaurin, like fatty acids in general, may be partly due to an 'uncoupling' effect of the plasma membrane (Stratford and Anslow, 1996). Wang and Johnson (1992) observed that in *Listeria* cells treated with monolaurin, the cytoplasmic contents appeared to separate from the cell envelope but the cell surface appeared to remain intact. In other cells, however, breakage of the cell envelope and leakage of cytoplasmic contents were observed.

- **Inhibition of enzymes**

Galbraith and Miller (1973b, c) proposed that fatty acids may affect the respiratory activity of cells by the inhibition of enzymes involved in oxygen uptake and inhibits the transport of amino acids into cells. It also has been shown that monolaurin affects neither secretion nor intracellular signalling and most likely acts through the inhibition of signal transduction (Ruzin and Novick, 1998; 2000). At lower concentrations, which do not significantly alter bacterial growth, monolaurin blocks the production of various exoenzymes and virulence factors, including protein A, alpha-hemolysin, β-lactamase, and toxic shock syndrome toxin 1 (TSST-1) in *Staphylococcus aureus* (Ruzin and Novick, 2000). The action of monolaurin is not restricted to *S. aureus*; it also blocks the induction of vancomycin resistance in another pathogen, *Enterococcus faecalis* (Ruzin and Novick, 1998).

1.4.2 Evaluation of the antimicrobial effect on the cell membrane

A number of assays have been developed to detect and quantify membrane damage.

- **Leakage of material absorbing at 260 nm**

Low molecular weight metabolites known to leak from cells include nucleotides and their component structures (purines, pyrimidines, pentose and inorganic phosphate), amino acids and inorganic ions. The presence of these materials in a suspension indicates damage to the cell at the membrane level and can be determined by their absorbance at 260 nm.

Several studies have implemented this method, called the OD_{260} assay, to evaluate the mode of action of antimicrobial compounds against a different range of microorganisms (Liu *et al.*, 2004; Oonmetta-aree *et al.*, 2006). O'Neill *et al.*, (2004) compared different assays for detection of agents causing membrane damage in *Staphylococcus aureus* and concluded that the OD_{260} assay should be sufficient for most purposes since it is effective, rapid and cheap to perform.

- **Ions**

Generally, the first sign of an increase in cytoplasmic membrane permeability is provided by leakage of the important intracellular solute potassium, and in recent years measurement of potassium loss from bacterial cells has successfully been used to detect membrane damage caused by phytochemicals present in tea tree oil (Cox *et al.*, 2000), oregano essential oil (Lambert *et al.*, 2001) and galangin (Cushnie and Lamb 2005; 2006).

Potassium and hydrogen can leak and be detected by ion electrodes. Ion-selective electrodes are used to monitor the formation of ion channels that affect the membrane potential in bacteria. Potentiometric measurements have several inherent advantages over spectrophotometric methods, i.e., simplified procedures, low costs and ease of continuous monitoring in a turbid cell suspension (Johnston *et al.*, 2003).

- **Measurement of ATP**

The leakage of low molecular weight compounds is frequently followed by leakage of larger cellular constituents [eg. adenosine triphosphate (ATP)] either as damage to the membrane increases or as soluble products are formed from the gradual breakdown of proteins and nucleic acids by the autolytic enzymes of the cell (Johnston *et al.*, 2003).

Cells can release nucleotides, such as ATP and UTP, in response to mechanical stress or biological activation. Leakage of intracellular ATP is the result of increased membrane permeability. Commercial kits are available that measure ATP leakage by fluorescence or luminescence methods.

O'Neill *et al.*, (2004) recommended that studies requiring maximum sensitivity and discrimination should employ the ATP assay.

- **Enzymatic assays**

Enzymatic assays are an aid to assess the damage of the cell membrane. Different assays are commercially available to determine different enzymes present in the cytoplasm of the cells which can be detected when the membrane has been disrupted. For example, the cytoplasmic enzyme lactate dehydrogenase is released into the cell culture medium if the membrane is damaged. Another assessment of membrane

permeabilization is done by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* into the culture medium using ONPG (O-nitrophenyl-β-D-galactopyranoside) as the substrate.

- **Fluorescent methods (BacLight assay)**

Conventional direct-count assays of bacterial viability are based on metabolic characteristics or membrane integrity. Fluorescent probes provide alternative methods for assessment of bacterial physiology (Haugland,1996; Breeuwer and Abee, 2000). Recently the Live/Dead® BacLight™ Bacterial Viability Kits sold by the Molecular Probes, Inc. have been used for bacterial enumeration and viability testing in various ways (Virta *et al.*, 1998; Bunthof *et al.*, 2001; Singh, 2006).

The Live/Dead® BacLight™ Bacterial Viability Kits utilize mixtures of SYTO® 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population - those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red.

- **Electron microscopy**

Cellular integrity can be evaluated as reflected by transmission electron microscopy. Using electron microscopy, any gross morphological changes that take place when cells are exposed to a lethal dose of antimicrobial can be observed. For example, (Skrivanova *et al.*, 2005) using electron microscopy photographs observed that there was no visible alteration in the cell wall structure and size of cells, but the separation of inner and outer membranes and complete cytoplasm disorganization in lauric acid-treated cells was apparent.

1.4.3 Phenotypic MicroArray to identify antimicrobials and their mechanism of action

Technologies that offer a cell-wide perspective are very useful in biology. O'Farrell (1975), and Fodor *et al.* (1993), initiated using two dimensional methods for protein analysis (proteomics), and for nucleic acid analysis (genomics), respectively. These technologies and subsequent improvements allow for global analysis of the important macromolecules of cells that convey the information flow from DNA to RNA to protein. However, the information initially encoded in the genome is ultimately displayed at the cellular level as cellular traits or phenotypes (Bochner *et al.*, 2001). Phenotype is defined as the observable physical or biochemical characteristics of an organism, as determined by both genetic makeup and environmental influences.

Phenotype MicroArrays (PM) is a recent technology that provides an analogous two-dimensional array technology for analysis of live cells to screen the response of an organism to thousands of chemical treatments simultaneously (Bochner, 1989).

Phenotype MicroArray (PM) are sets of phenotypic assays performed in 96-well microplates with chemicals included to create unique culture conditions after rehydration. Microplates in the PM set are organized into functional groups as labelled in Figure 1.7. Biolog, Inc. (Hayward, CA) currently markets 20 phenotype microarrays that fall into different 'modes of action' categories. These categories include different sources (carbon, nitrogen, sulfur, phosphorus), nutritional supplements, nitrogen utilization, osmotic sensitivity, toxicity, pH, inhibitors, and chemical sensitivity.

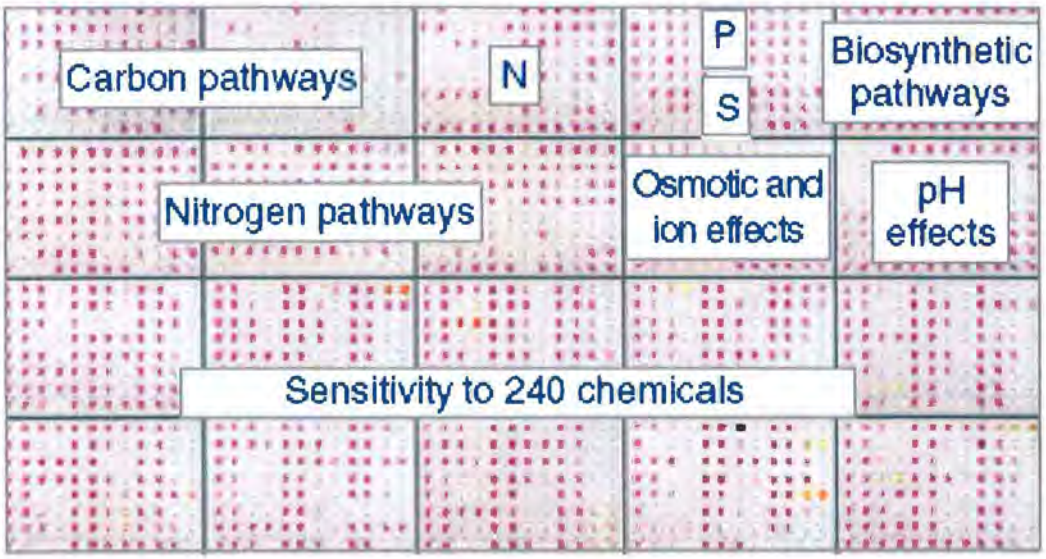


Figure 1.7 The phenotypic assays in the PM set for bacteria (Bochner, 2009).

Assays of carbon, nitrogen, sulphur and phosphorus metabolism provide information about which metabolic pathways are present and active in the microbial cells. Assays of ion, pH, and chemical sensitivities provide information on stress and repair pathways that are present and active in cells (Bochner, 2009).

For novel antimicrobials, a large effort is often needed to determine their mechanism of action (MOA). Genetic and biochemical approaches are labour intensive

and may fail to discover an MOA. Phenotype MicroArray (PM) technology can be used to screen and identify novel antimicrobials and to further infer a MOA of an inhibitor (Wiater *et al.*, 2004; Biolog, 2007).

Antimicrobials can be added to cells prior to inoculation into PMs. By looking at the phenotypes altered by the antimicrobial compound one can determine the physiological functions in the cell that are affected. This information will indicate: (1) the site and/or mode of action of a drug, (2) whether the antimicrobial compound is specifically hitting one target or whether it is interfering also with other cellular processes and therefore likely to cause side-effects, (3) potentially beneficial as well as detrimental interactions with other antimicrobial compounds (many of the phenotypes in the PMs test for increased sensitivity or resistance to existing drugs).

Assays are initiated by inoculating all wells with cell suspensions, either alone or with the test compound. The assays are respiration based and colorimetric based on reduction of a tetrazolium dye. The variable level of purple colour indicates that the cells are metabolically active and respiring in some wells but not others. A kinetic response curve, which parallels microbial growth, is generated so that a growth parameter can be extracted (Bochner, 2009). The software can automatically compare two or more cell lines over hundreds to thousands of phenotypes.

The interaction of the microorganisms' biochemical machinery and inhibitory chemicals dictates the growth characteristics. The Biolog system uses OmniLog data capture software to exploit growth characteristics to generate a high-resolution isobologram, which display the interactions that two inhibitors have on microbial growth. Typically, isobolograms are generated by mixing two chemical inhibitors in

different proportions at fractional minimal inhibitory concentrations (MICs) and then determining if those combinations prevent microbial growth (Figure 1.8).

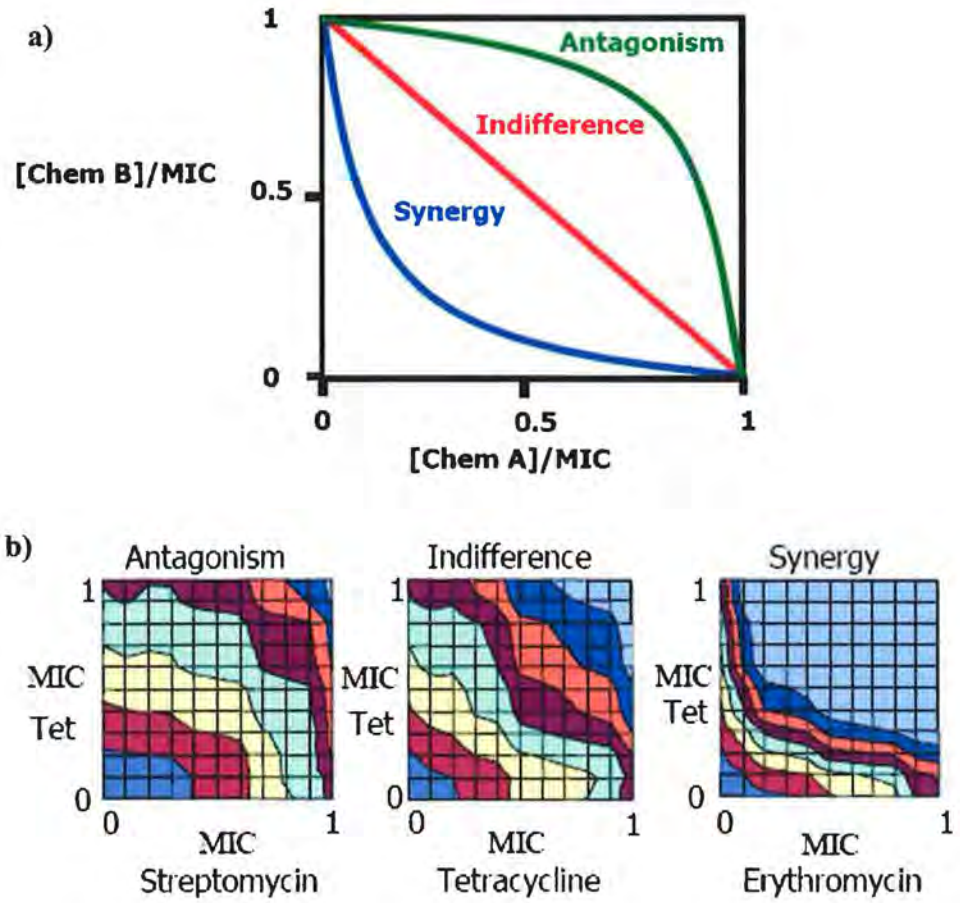


Figure 1.8 Isobolograms to determine chemical interactions a) Classical and b) Biolog OmniLog (Biolog, 2007)

The synergy and antagonism magnitudes taken from such isobolograms can be used to cluster chemicals with known mechanisms of action (Figure 1.9). Novel inhibitors that cluster within a group of inhibitors are inferred to have the same MOA (Biolog, 2007).

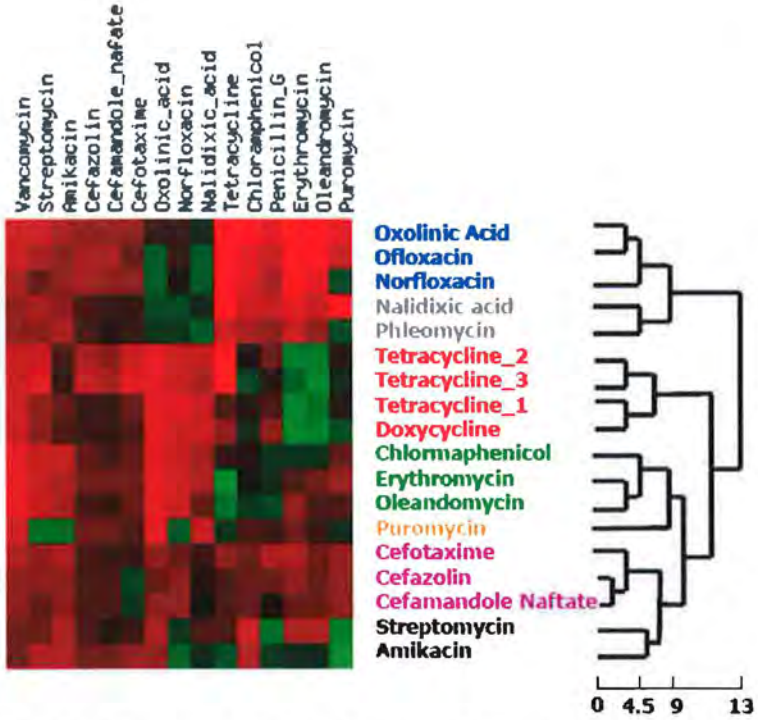


Figure 1.9 Biolog Isobologram data used to group known antibacterials (Biolog, 2007).

1.5 SYNTHESIS OF CARBOHYDRATE FATTY ACID DERIVATIVES

Carbohydrates are the most widely distributed, naturally occurring compounds on Earth. Their general formula is $C_n(H_2O)_n$. Carbohydrates are highly functionalised small organic molecules. Synthetically, they can be reduced to give sugar alcohols, oxidized to give sugar acids, substituted at one or more of the hydroxyl groups or derivatized at the hydroxyl groups.

Carbohydrates represent an under-utilised source of diversity; this is partly due to their multiple hydroxyl groups which make carbohydrate molecules a particularly challenging target for regioselective synthetic modifications. Different degrees of substitution result in different physicochemical properties, which in turn will influence/affect their function in different applications. Control of regioselectivity of carbohydrates is very important when developing a route to carbohydrate derivatives. In order to develop a chemical synthesis yielding pure, regiodefined carbohydrate fatty acid derivatives it is important to first consider carbohydrate structure and, usually, protecting group strategies for polyhydroxylated molecules.

1.5.1 Structure of carbohydrates: focus on glucose and related sugars.

The most basic carbohydrate units are called monosaccharides. The most common types of structures are the aldoses. Glucose is an example, containing six carbons, known as hexose. Aldoses consist of a linear carbon chain with an aldehyde group at C-1, a varying number of carbon atoms which are secondary alcohols (for D-glucose are at C-2, 3, and 4) and a primary alcohol at the other end of the chain (for D-glucose is at C-6).

The aldehyde group of glucose can react intramolecularly with a hydroxyl group to give a hemiacetal, forming a heterocyclic ring with an oxygen bridge between two carbon atoms. The hydroxyl of this hemiacetal is known as the anomeric hydroxyl. Rings with five and six atoms are called furanose and pyranose forms, respectively, and exist in equilibrium with the straight-chain form (Figure 1.10.).

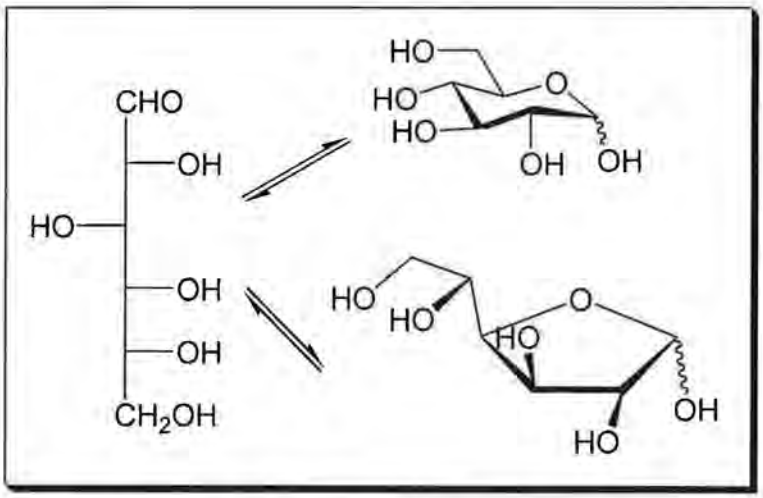


Figure 1.10: Pyranose and furanose of glucose

During the conversion from straight-chain form to cyclic form, the carbonyl carbon (anomeric carbon), becomes a chiral center with two possible configurations. The resulting possible pair of stereoisomers are called anomers. The two possible configurations for glucopyranoside are shown (Figure 1.11). The reaction of glucose, at the anomeric position, with an alcohol forms a glycoside, for example a methyl glucopyranoside or a dissacharide. This glycosylation reaction locks the configuration of the anomeric centre and prevents further equilibrium interconversions.

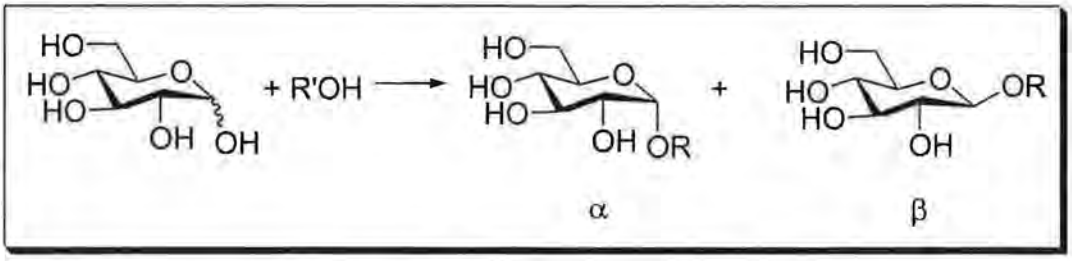


Figure 1.11: Glycosylation reaction producing α and β anomers of glucose.

Each of the carbon atoms that bear secondary alcohols have four different groups attached and are stereogenic (or chiral) centres. Each asymmetric centre has two possible configurations. Because of this asymmetry, a number of isomers may exist for any given monosaccharide formula. Glucopyranoside, its C-4 epimer galactopyranoside and its C-2 epimer mannopyranoside are an example (Figure 1.12).

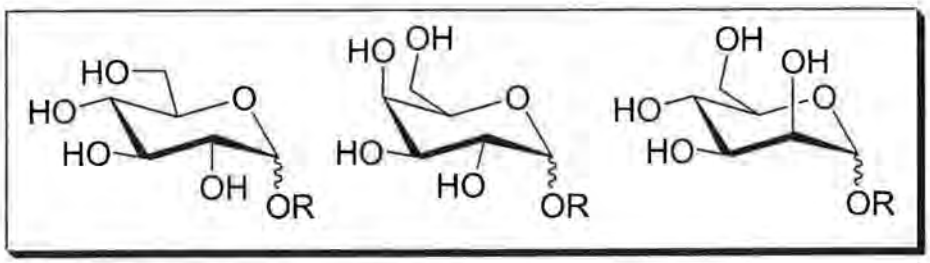


Figure 1.12: Glucopyranoside, galactopyranoside and mannopyranoside.

These can be used as scaffolds from which many products can be obtained after the reaction of one or more hydroxyl groups. Monosaccharides can be linked together in almost limitless ways to form oligo- and polysaccharides.

1.5.2 Protecting groups for a synthetic strategy for carbohydrates

Although it is possible to synthesise pure oligosaccharides in the laboratory, the regioselective protection of hydroxyl groups and the stereoselective assembly of glycosidic bonds present a number of challenges (Wang *et al.*, 2007). When a chemical reaction is to be carried out selectively at one reaction site in a multifunctional compound, other reactive sites are often temporarily blocked, so as not to react. In carbohydrates such as glucose, the strategic placement of protective groups that mask hydroxyl groups are frequently required because of the polyhydroxylated structure.

The ability to remove one protecting group in the presence of others is a key feature, moreover the steric and electronic nature of the protecting groups are also important (Bartolozzi and Seeberger, 2001).

Protecting groups should satisfy several important criteria. They should be cheap or readily available, be easily introduced and formed in good yield, be stable to subsequent reaction conditions, be selectively removed under appropriate conditions and finally, the by-products of the deprotection should be easily separated from the substrate (Kocienski, 2000).

Protecting groups of relevance to this study are:

- *Triisopropylsilyl Ether*

The triisopropylsilyl ether (TIPS) group is a member of the silyl ether protecting groups. Due to its bulky nature it is selective for the primary hydroxyl. Although many methods are available for forming silyl ethers, there are two common strategies: reaction of the alcohol with a silyl chloride with an amine base at room temperature and reaction of the alcohol with a silyl triflate with a hindered amine base at low temperature. One extremely reliable and rapid procedure is the Corey protocol in which the alcohol is

reacted with a silyl chloride and imidazole at high concentration in DMF (Corey and Venkateswarlu, 1972).

TIPS groups (Figure 1.13) are relatively stable towards basic hydrolysis, oxidation, reduction and column chromatography (Kocienski, 2000).

Reactions with acids or fluorides such as tetra-n-butylammonium fluoride remove the silyl group when protection is no longer needed.

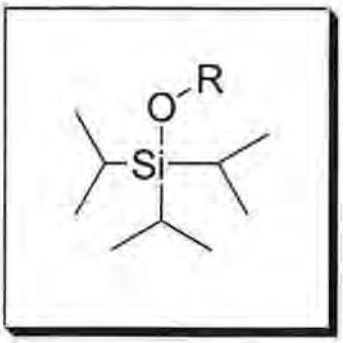


Figure 1.13. Triisopropylsilyl ether protected alcohol

- *Benzyl Ethers*

Benzyl ethers (Figure 1.14, abbreviated Bn) are often used for the protection of free primary and secondary hydroxyl groups.

The most common method for preparing benzyl ethers is the Williamson ether synthesis where the alcohols react with benzyl bromide and a strong base such as sodium hydride.

Benzyl ethers are stable to a wide range of aqueous acidic and basic conditions, and they are not readily attacked by most metal hydride reducing agents or mild oxidising agents (Kocienski, 2000).

They are most readily cleaved under catalytic hydrogenation with a catalyst such

as palladium on carbon. Under these conditions most other groups, including esters, are stable.

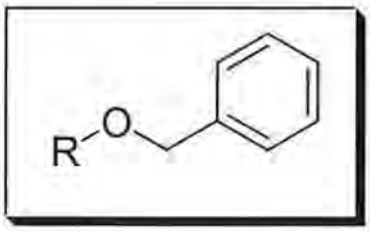


Figure 1.14. Benzyl ether protected alcohol

- *Para-methoxybenzyl ether*

The para-methoxybenzyl (PMB) ether (Figure 1.15) has found application in organic synthesis as a protecting group for primary and secondary alcohols since it may be selectively removed in the presence of many other groups, including ethers.

Sodium alkoxides prepared from primary and secondary alcohols and sodium hydride in DMF or DMSO react with PMB chloride at room temperature to give the PMB ethers in good yield (Kocienski, 2000).

PMB groups are less stable to acid than benzyl groups, but they can be removed under specific conditions which do not affect benzyl ethers or silyl ethers and therefore find use in the synthesis of functionally complex molecules (Kocienski, 2000).

PMB can be removed by acid, hydrogenolysis, or oxidation. They may be cleaved selectively by oxidation with one electron oxidising agents such as ceric ammonium nitrate (CAN).

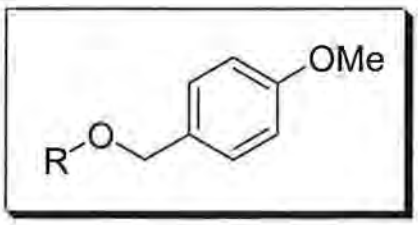


Figure 1.15. Para-methoxybenzyl ether protected alcohol

- *Benzylidene group*

Benzylidene acetals (Figure 1.16) are frequently used in carbohydrate chemistry for selective 4,6-di-*O*- protection in hexopyranosides (Lindhorst, 2007), which is particularly useful since it allows manipulation of the remaining hydroxyl groups (Chambers *et al.,* 2003). Moreover, 4,6-di-*O*-benzylidene protection is particularly desirable since regioselective reductive cleavage of the benzylidene group (Garreg, 1997), which may be performed at any later point in a synthetic sequence, allows selective access to either the 4- or 6-hydroxyl groups as desired.

There are only two methods generally used to prepare benzylidene acetals: reaction of a diol with benzaldehyde in the presence of a protic acid or Lewis acid (usually $ZnCl_2$) or reaction of the diol with benzaldehyde dimethyl acetal in the presence of an acid catalyst (p-toluenesulfonic acid). They are stable to most strong bases, mild oxidants and metal hydrides. Acidic hydrolysis is an efficient means of cleaving them.

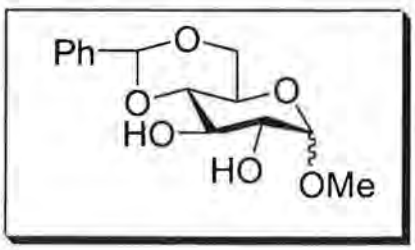


Figure 1.16. Methyl 4,6-di-O-benzylidene-D-glucopyranoside

1.5.3 Monitoring reactions with thin layer chromatography (TLC)

Thin layer chromatography (TLC) is one of the most popular and widely used analytical techniques. Because of its simplicity, wide applications to a great number of samples, speed and relatively low cost, it is often used for monitoring chemical reactions and for the qualitative analysis of reaction products (Higson, 2006).

Thin layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of stationary phase, usually silica gel, aluminium oxide, or cellulose. After the sample has been applied on the plate, a solvent or solvent mixture (called the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved (Higson, 2006). Different compounds in the sample mixture travel at different rates due to the differences in the partition co-efficient between stationary phase and mobile phase. By varying the mobile phase, the separation of components (measured by the R_f value) can be adjusted. The R_f value is a convenient way to express the position of a substance on a developed plate. It is calculated as the ratio:

$$Rf = \frac{\text{distance of compound from origin}}{\text{distance of solvent front from origin}}$$

As the compounds being separated may be colourless, several methods exist to visualize the spots. Often a small amount of a fluorescent compound is added to the adsorbent that allows the visualization of spots under a black light (UV_{254}). Several colour stains exist into which the TLC plate is dipped or which are sprayed onto the plate. These react with the analyte compounds and coloured spots appear on the TLC plate.

1.5.4 General synthesis of esters and ethers

Esters are commonly synthesised by reaction of an alcohol with an activated carboxylic acid derivative (Figure 1.17). They can also be formed by acid or enzyme catalysed reaction between an alcohol and a carboxylic acid.

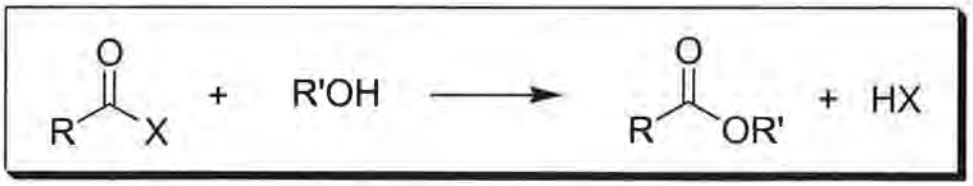


Figure 1.17. General synthesis of esters using an activated carboxylic acid

Ethers are commonly formed by the reaction of an alcohol with an alkyl halide. (Figure 1.18)

Figure 1.18. General synthesis of ethers.

1.6 OBJECTIVES

1.6.1 General objective

This study was concerned with the synthesis of carbohydrate fatty acid derivatives for evaluation as antibacterial agents, with a view to examining the effect of variation of the hydrophilic moiety on antimicrobial activity. Studies focused on lauric and caprylic acids, and their ester and ether conjugates were synthesised to allow comparative studies.

Additionally, other natural compounds including bacteriocins and essential oil components were screened for antimicrobial activity towards possible combination strategies and potential further use in synthesis or modification studies.

1.6.2 Specific objectives

- To synthesize pure, regiochemically defined monosaccharide mono-fatty acid esters, and their corresponding ethers as potential antimicrobial compounds (AMC).
- To screen carbohydrate based AMC for quantitative assessment of antimicrobial activity compared to structurally similar commercial products.
- Compare the effect of the AMC on Gram positive and Gram negative pathogens, and spoilage microorganisms.
- Build up knowledge allowing investigation of any structure/activity relationship and mode of action of these compounds.
- To investigate other natural compounds towards possible combination strategies.

2. MATERIALS AND METHODS

2.1 BACTERIA AND GROWTH CONDITIONS

Tests were performed using pathogenic and spoilage microorganisms. Bacterial strains used in this study are listed in Table 2.1.

Stock cultures were maintained in tryptic soy broth (TSB, Sharlau Chemie, Spain) or De Man, Rogosa and Sharpe (MRS broth, Sharlau Chemie, Spain) broth for *Lactobacillus,* supplemented with 20% glycerol and stored at -70°C. Cultures were routinely grown by subculturing a loopful of stock culture into 9 mL TSB and incubating at 35°C for 18-24 hours, except for *Pseudomonas* spp. and *Lactobacillus* spp. which were incubated at 30°C. All cultures were then maintained on tryptic soy agar (TSA, Sharlau Chemie, Spain) or MRS agar (MRS agar, Sharlau Chemie, Spain) plates at 4°C and were discarded after subculturing for 2 weeks. Working cultures were prepared by inoculating a loop of pure culture into TSB and incubating at the optimum temperature for each strain for 18 hours.

Table 2.1. List of bacterial strains tested

Strain	Reference [a]	Source / Comments
Gram-positive bacteria		
Lactobacillus plantarum	ATCC 8014	Fermented food
Lactobacillus sakei	ATCC15521	Moto, starter of sake
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
Staphylococcus aureus	ATCC 25923	Clinical isolate
Staphylococcus aureus	NCTC 1803	Mammal, ovine gangrenous mastitis
Staphylococcus aureus	ATCC 33591	Methicillin resistant
Staphylococcus aureus	ATCC 33592	Blood, Gentamicin- and methicillin-resistant
Staphylococcus aureus	ATCC 43300	clinical isolate, F-182, Methicillin- and oxacillin-resistant
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 aeruginosa	ATCC 27853	Blood culture
Pseudomonas fluorescens	*	Lettuce
Pseudomonas putida	*	Lettuce

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

2.2 BACTERIAL CALIBRATION CURVES

Bacterial calibration curves were constructed to equate absorbance values with cell growth and density in Log_{10} CFU/mL.

Bacterial strains were grown overnight in TSB at 35°C. A bacterial suspension was prepared in an API ampoule with saline solution (NaCl 0.85%, BioMérieux, France) to obtain a concentration equal to a McFarland standard of 0.5, equivalent to $1x10^8$ CFU/mL using a Densimat photometer (BioMérieux, SA, France). The bacterial suspension was diluted 1:100 in sterile TSB broth to obtain approximately $1x10^6$ CFU/mL. This inoculum (100 µL) was added to replicate wells of a 96-well plate containing 100 µL of fresh TSB. The plates were incubated at 35°C for 18 hours in a microtiterplate reader (PowerWave microplate Spectrophotometer, BioTek). Subsequently, 100 µL aliquots were taken at regular intervals and serially diluted in sterile saline solution (SS) (Sodium chloride, Sigma, USA) and the appropriate dilutions were spread plated on TSA. Plates were incubated at 35°C and after 18-24 hours colony forming units were counted. Absorbance values were plotted against the Log_{10} CFU/mL to obtain a logarithmic trendline. The equations and the R^2 values were determined.

2.3 TEST COMPOUNDS

2.3.1 Fatty acid and monoglyceride standards

The saturated fatty acids lauric acid (LA – C12) and caprylic acid (CA - C8) as well as their corresponding monoglycerides; monolaurin (ML) and monocaprylin (MC) (Sigma-Aldrich ~99% purity) were investigated and used as standards in this study.

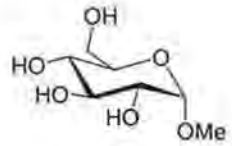
Standard stock solutions (100 mM) of the fatty acids and monoglycerides were prepared in sterile hydroalcoholic diluent (ethanol-distilled water, 1:1) and stored at -20°C. Stock solutions were diluted in TSB to obtain working concentrations. An overview of the concentration ranges used for the standards in the antimicrobial assay is given in Table 2.2. The highest final concentration of ethanol (5% and 10%), corresponding to the highest concentration of compound used (10mM for the Gram positive and 20mM for the Gram negative bacteria) had a negligible effect on bacterial viability.

Table 2.2. Range of concentrations used for the standard compounds (μg/mL).

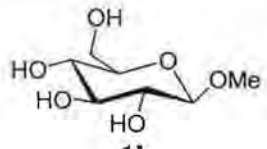
mM	Lauric acid (LA)	Monolaurin (ML)	Caprylic acid (CA)	Monocaprylin (MC)	Ethanol final concentration
100	20032	27440	14422	21829	50 %
20	4006.4	5488	2884.4	4365.8	10 %
10	2003.2	2744	1442.2	2182.9	5 %
5	1001.6	1372	721.1	1091.5	2.5 %
2.5	500.8	686	360.6	545.7	1.25 %
1.25	250.4	343	180.3	272.9	0.63 %
0.63	125.2	171.5	90.1	136.4	0.31 %
0.31	62.6	85.75	45.1	68.2	0.16 %
0.16	31.3	42.9	22.5	34.1	0.08 %
0.08	15.7	21.4	11.3	17.1	0.04 %
0.04	7.8	10.7	5.6	8.5	0.02 %
0.02	3.9	5.4	2.8	4.3	0.01 %

2.3.2 Carbohydrate fatty acid (CFA) derivatives

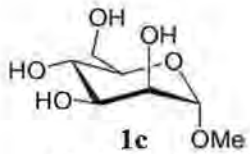
Chemical synthesis was performed according to Smith *et al.*, (2008). More information on the synthesis of compounds can be found in Chapter 3, Section 3.1 and the methodology is detailed in Appendix 1.1 (Smith *et al.*, 2008 Carbohydrate Research paper). An overview of the fatty acid derivatives synthesized and used in the antimicrobial assay is given in Figure 2.1. Test stock solutions (100 mM) of the fatty acid derivatives were prepared in sterile hydroalcoholic diluent (ethanol-distilled water, 1:1) and stored at -20°C. Test stock solutions were diluted in TSB or appropriate media to obtain working concentrations.



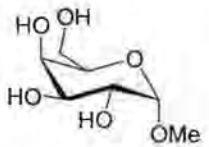
1a
Methyl α-D-glucopyranoside



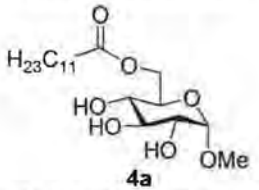
1b
Methyl β-D-glucopyranoside



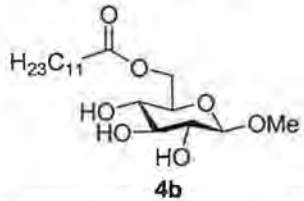
1c
Methyl α-D-mannopyranoside



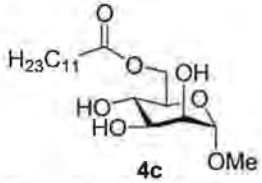
1d
Methyl α-D-galactopyranoside



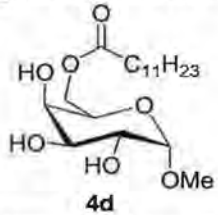
4a
Methyl 6-O-lauroyl-α-D-glucopyranoside



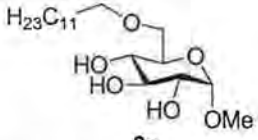
4b
Methyl 6-O-lauroyl-β-D-glucopyranoside



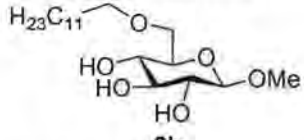
4c
Methyl 6-O-lauroyl-α-D-mannopyranoside



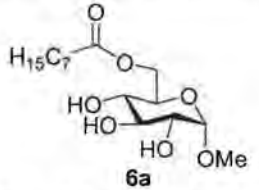
4d
Methyl 6-O-lauroyl-α-D-galactopyranoside



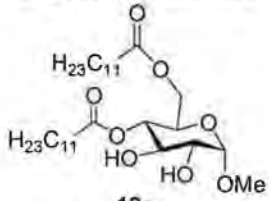
9a
Methyl 6-O-dodecanyl-α-D-glucopyranoside



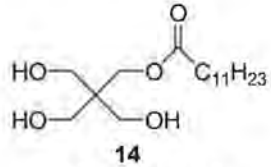
9b
Methyl 6-O-dodecanyl-β-D-glucopyranoside



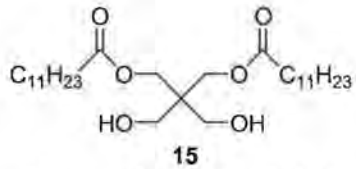
6a
Methyl 6-O-octanoyl-α-D-glucopyranoside



12a
Methyl 4,6-di-O-lauroyl-α-D-glucopyranoside



14
Monolauroyl pentaerythritol



15
Dilauroyl pentaerythritol

Figure 2.1 Structures of the novel carbohydrate fatty acid derivatives and non-carbohydrate polyhydroxylated esters synthesized and investigated.

2.4 ANTIMICROBIAL ACTIVITY OF CFA DERIVATIVES

The minimal inhibitory concentration (MIC) of carbohydrate fatty acid derivatives and standards was determined using an absorbance based broth microdilution assay in sterile 96-well plates (Sarstedt Ltd., Leicester, UK) in a final volume of 200 µL as follows.

2.4.1 Inoculum preparation.

Bacterial strains were grown overnight in TSB at 35°C. A bacterial suspension was prepared in an API ampoule with saline solution (NaCl 0.85%, BioMérieux, France) to obtain a concentration equal to a McFarland standard of 0.5, equivalent to $1.5x10^8$ CFU/mL using a Densimat photometer (BioMérieux, SA, France). The suspended inoculum was diluted 1:100 in sterile TSB broth to obtain approximately $1x10^6$ CFU/mL. The diluted inoculum (100 µL) was added to each appropriate well.

2.4.2 Test compounds preparation.

Standards and carbohydrate fatty acid derivatives were prepared as previously described in sections 2.3.1 and 2.3.2. Freshly prepared solutions of the test compounds were serially diluted twofold in sterile culture media to a final volume of 100 µL in the 96-well plate (Table 2.3). The following controls were used in the microplate assay for each organism and test compound:

- **Blank**: uninoculated media without test compound to assess changes in liquid media during the experiment.
- **Negative control**: uninoculated media containing only the tested compound to assess background noise.

- **Positive control 1**: inoculated media without compound to evaluate the microbial growth under optimal conditions.
- **Positive control 2**: inoculated media without compound but including the corresponding sugar to evaluate any effect of the sugar alone.
- **Positive control 3**: inoculated media without compound but containing appropriate amount of ethanol to account for a possible antagonist or synergistic activity of the alcohol used to dissolve the test compound.

Table 2.3. Carbohydrate fatty acid derivatives concentrations (μg/mL)

mM	α and β lauric ester	α and β lauric ether	α caprylic ester	Ethanol final concentration
100	37650	36250	32030	50 %
20	7530	7250	6406	10 %
10	3765	3625	3203	5 %
5	1882.5	1812.5	1601.5	2.5 %
2.5	941.3	906.3	800.8	1.25 %
1.25	470.6	453.1	400.4	0.63 %
0.63	235.3	226.6	200.2	0.31 %
0.31	117.7	113.3	100.1	0.16 %
0.16	58.8	56.6	50.0	0.08 %
0.08	29.4	28.3	25.0	0.04 %
0.04	14.7	14.2	12.5	0.02 %
0.02	7.4	7.1	6.3	0.01 %

The inoculation scheme yielded final concentrations of each microorganism estimated to be $5x10^5$ CFU/mL and chemical compounds ranging from a high test

dilution of 1:2 (inoculum mixed with 100 µL of diluted chemical compound) to a low test dilution of 1:256. Each concentration was assayed in duplicate.

2.4.3 Broth microdilution assay.

The 96-well plates were incubated for 18 hours in a microtiterplate reader (PowerWave microplate Spectrophotometer, BioTek) at 35°C, except for *Pseudomonas* spp. which were incubated at 30°C, and bacterial growth was monitored by measuring the optical density (OD) at 600 nm for each well every 20 minutes with 20 seconds agitation before each OD measurement. Each experiment was replicated three times.

2.4.4 Data analysis.

2.4.4.1 MIC determination

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 hours. 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 changes in the media.

2.4.4.2 IC_{50} calculation

The IC_{50} or the half maximal inhibitory concentration represents the concentration of an antimicrobial that is required for 50% inhibition of its target microorganism. The IC_{50} was calculated for selected compounds. The reduction in optical density was determined by subtracting the OD_{600} of the culture at 18 hours containing antimicrobial compounds at a range of concentrations, from the OD_{600} of the negative control. The data was converted to log CFU/mL using the calibration curves previously constructed.

The percentage inhibition was then calculated against the positive control 1 results. A dose-response curve was constructed for the compounds for a concentration range that did not show a 100% inhibition. The slope of the linear portion of the line was calculated and the IC_{50} was then calculated using the equation obtained.

2.4.4.3 Increase in Lag time estimate.

Lag phase was defined as the time required for the culture to reach a change in optical density (ΔOD) of 0.10. This change in OD was calculated using the Gen5™ software supplied with the microplate spectrophotometer used for sample assays. The increase in lag phase was determined as the time the culture containing antimicrobial compound took to record an increase in ΔOD of 0.10 minus the time that the positive control 1 took to record the same increase in ΔOD of 0.10.

2.4.4.4 Maximum specific growth rate (μmax)

The maximum growth rate was also calculated using the Gen5™ software. The μ_{max} was determined from the slope of the regression equation from the linear portion of the log plot during early exponential phase.

2.4.4.5 Statistical analysis of results

The results for MIC determinations were subjected to statistical analysis. Statistical differences were analysed using ANOVA followed by LSD testing at $p < 0.05$ level using SPSS software, Version 15 (IBM).

2.5 EFFECT OF pH AND TEMPERATURE ON THE FATTY ACIDS AND MONOGLYCERIDES ACTIVITY

Lauric acid and monolaurin standards were used to assess the potential effect of temperature and pH on antimicrobial compound potencies.

2.5.1 Test conditions.

A total of 24 combinations of temperature and pH were examined. Four temperatures were used with different incubation times to simulate different storage conditions:

- 35°C x 18h (Optimal bacterial growth)
- 22°C x 48h (Ambient)
- 10°C x 120h (Temperature abuse for refrigeration)
- 4°C x 120h (Recommended refrigeration)

The pH values used (4, 4.5, 5, 5.5, 6, and 7) were used to reflect different pH values found in a range of common food products.

2.5.2 Inoculum and test compounds preparation.

TSB was used as the basal medium for all experiments in broth. The pH levels of the media were adjusted to the appropriate values with 1 M HCl and were measured before and after autoclaving using a digital pH meter (Orion 420A) with a glass pH electrode (Orion). Measurement of the pH was also determined when the fatty acid or monoglyceride were added. Bacterial strains were prepared as described previously (Section 2.4.1.) Concentrations of lauric acid and monolaurin corresponding to the 0.5

MIC, MIC and 2x MIC were prepared in the pH adjusted broths as previously described in section 2.3.1.

2.5.3 Assay.

The 96-well plates were inoculated and covered with Parafilm to avoid dehydration. The plates were stored in controlled temperature incubators for the specific incubation times. Growth was monitored periodically by recording the turbidity of the medium using an automated microplate reader (PowerWave microplate Spectrophotometer, BioTek).

2.6 DEGRADATION OF CFA DERIVATIVES MONITORED BY THIN LAYER CHROMATOGRAPHY (TLC)

To determine the fate of the CFA derivatives in bacterial cultures, samples of *S. aureus* ATCC 25923 growing in the presence of CFA derivatives were analyzed by TLC according to Ruzin and Novik, 2000.

CFA derivatives were added to bacterial cultures at their corresponding MIC values. Samples were collected at different time points: 0, after 5, 15, 30 60 and 120 minutes, centrifuged at 10.000 *g* for 15 minutes and a sample of the supernatant (8 μL) was spotted onto TLC plates (Whatman silica gel 60A TLC plates (20 x 20, with preadsorbent area). Emulsions of LA (30 µg/mL) and CFA derivative (MIC concentration) in TSB broth were used as controls. Plates were air dried and developed with hexane-diethyl ether-methanol (70:20:10), air dried, baked at 100°C for 10 min, and sprayed with a 0.025% (wt/vol) solution of Coomassie R-250 in 20% (vol/vol) methanol until fatty acids were visible as white spots on a blue background.

Even though the samples were well separated during loading on the plate (1 cm), after development of the TLC plate, lateral dragging was observed. No distinct well formed spots could be observed by this method. In order to obtain better analytical data, a mini workup was performed on the broth sample to extract the analytes from the aqueous broth. Also the plates were loaded with 2 cm distance between samples. No evidence of lauric acid was found on the plate, possibly due to low concentration.

After the first attempt of Ruzin and Novick's protocol failed, it was decided to develop a new method suitable for the compounds generated in this study. A solvent system which gave rise to suitable mobility to all compounds for analytical purposes and a staining system to visualize them was required. The strategy first involved the use of pure compounds and subsequently after determining a suitable stationary phase, the mobile phase and stain, would be applied to the broth samples.

The different solvent systems and stains tested are listed below:

Stationary phase

- Silica gel TLC plates on aluminum support without preadsorbent area (Fluka)

Mobile phase

- Hexane:diethyl ether (80:20)
- Hexane:diethyl ether: methanol (70:25:5)
- Petroleum ether:diethyl ether:acetic acid (90:10:2)
- Pure hexane
- Pure ethyl acetate
- Ethyl acetate:petroleum ether (1:1)
- Ethyl acetate:petroleum ether (1:3)

Stains

- Solution of vanillin in pure ethanol with sulfuric acid 3% (wt/v)
- Sulfuric acid and water (50:50)
- Iodine vapors

2.7 EFFECT OF CFA DERIVATIVES ON THE CELL MEMBRANE

The laurate glucopyranoside derivatives (compounds **4a**, **4b**, **9a** and **9b**; Figure 2.1) were selected for the mode of action studies in order to assess the influence of structural differences in closely related compounds. These focussed on the nature of the fatty acid carbohydrate bond (ester or ether) as well as the anomeric configuration (alpha or beta) of the sugar.

2.7.1 Leakage of cellular compounds assay

2.7.1.1 Inoculum preparation

Bacterial strains were cultured in TSB and incubated at 35°C for 18-24 h. After incubation, bacteria were harvested by centrifugation at 10,000 *g* for 10 min at 4°C, supernatant was discarded and the cells were washed twice with PBS; pH 7.4 (Sigma). Cell suspensions were adjusted so that the optical density at 620 nm (OD620) of a 1-in-100 dilution was 0.3, which corresponded to ~$3x10^{10}$ CFU/mL. Final concentration of cells was approximately 10^9 CFU/mL.

2.7.1.2 Leakage of cellular compounds assay

Standards, CFA derivatives and nisin were added to bacterial suspensions at 4x MIC. Suspensions were incubated at 35°C in a water bath. Samples of 1.5 ml were removed at time 0, and after 15, 30, 45 and 60 minutes; and then centrifuged at 10,000 *g* for 10 minutes at 4°C. Following this, 200 µL of supernatant from each treatment were added in triplicate to wells of a 96-well plate (Costar) and absorbance values at 260 nm were recorded using a UV spectrophotometer (Synergy, Bio-Tek). The following controls were included: a bacterial suspension in sterile PBS without antimicrobial

agents as the negative control; Fatty acid and monoglyceride control: inoculated PBS containing LA or ML; Positive control: Nisin. Where applicable, independent readings were also taken in the presence of antibacterial agents only to enable corrections for background contributions.

2.7.2 Modified Live/Dead BacLight assay

2.7.2.1 Inoculum preparation

A culture of *Staphylococcus aureus* ATCC 25923 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 10–15 minutes. The supernatant was removed and the pellet resuspended in 2 mL of 0.85% NaCl. One mL of this suspension was added to each of two 30–40 mL centrifuge tubes containing either 20 mL of 0.85% NaCl (for live bacteria) or 20 mL of 70% isopropyl alcohol (for killed bacteria). Both samples were incubated at room temperature for 1 hour, mixing every 15 minutes and then pelleted by centrifugation at 10,000 × g for 10–15 minutes. The pellets were resuspended in 20 mL of 0.85% NaCl and centrifuged again as in the previous step. Both pellets were then resuspended in separate tubes with 10 mL of 0.85% NaCl in each. Optical density of the bacterial suspensions was adjusted to yield ~0.30 at OD_{670}. Five different proportions of *S. aureus* ATCC 25923 (Table 2.4) were mixed in glass culture tubes. The total volume of each of the five samples was 2 mL.

Table 2.4 Preparation of live- and dead-cell suspensions.

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0 2	0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

2.7.2.2 Staining

To assess membrane damage, the Live/Dead® BacLight viability kit from Molecular Probes, Inc. (Eugene, Oreg.) was used as described by Hilliard *et al.*, (1999). 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 the same proportions. 100 µL of each of the bacterial cell suspension mixtures were added in triplicate into separate wells of a 96-well flat-bottom microplate (Nunc, black). 100 µL of the 1X working stain solution were pipetted to each well and mixed thoroughly. The microtiter plate was then incubated at room temperature in the dark for 15 minutes.

2.7.2.3 Fluorescence measurements

With the excitation wavelength centered at about 485 nm, the fluorescence intensity was measured at a wavelength centered at about 530 nm (emission 1; green) for each well of the entire plate. With the excitation wavelength still centered at about 485 nm, the fluorescence intensity was measured at a wavelength centered about 630 nm (emission 2; red) for each well of the entire plate. The data was analysed by dividing the

fluorescence intensity of the stained bacterial suspensions (Fcell) at emission 1 by the fluorescence intensity at emission 2.

$$G/R\ Ratio = F\ cell,em1/F\ cell,em2$$

The RatioG/R was plotted versus percentage of live cells in the *S. aureus* suspension.

2.7.3 BacTiter-Glo™ assay

The BacTiter-Glo™ Microbial Cell Viability Assay (Promega) is a method for determining the number of viable bacterial cells in culture based on quantitation of the ATP present, as an indicator of metabolically active cells. 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 *S. 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 and cells without compound were used as ATP positive control. The microtiter plate was incubated at 37°C for 5 hours. One hundred microliters of the culture was taken from each well, and was 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).

2.8 PHENOTYPE MICROARRAY ™

Phenotype MicroArray technology was used to assess the differences or changes in bacterial growth and metabolic activity between untreated control cells and cells treated with selected CFA derivatives; as well as their interactions with known antimicrobials, to further elucidate possible antimicrobial mechanisms of action. Figure 2.2 shows the experimental stages of the Phenotype MicroArray (PM) technique. For this study, *L. monocytogenes* ATCC 7644 was used. The CFA derivatives selected were alpha ester (**4a**), alpha ether (**9a**) and beta ether (**9b**).

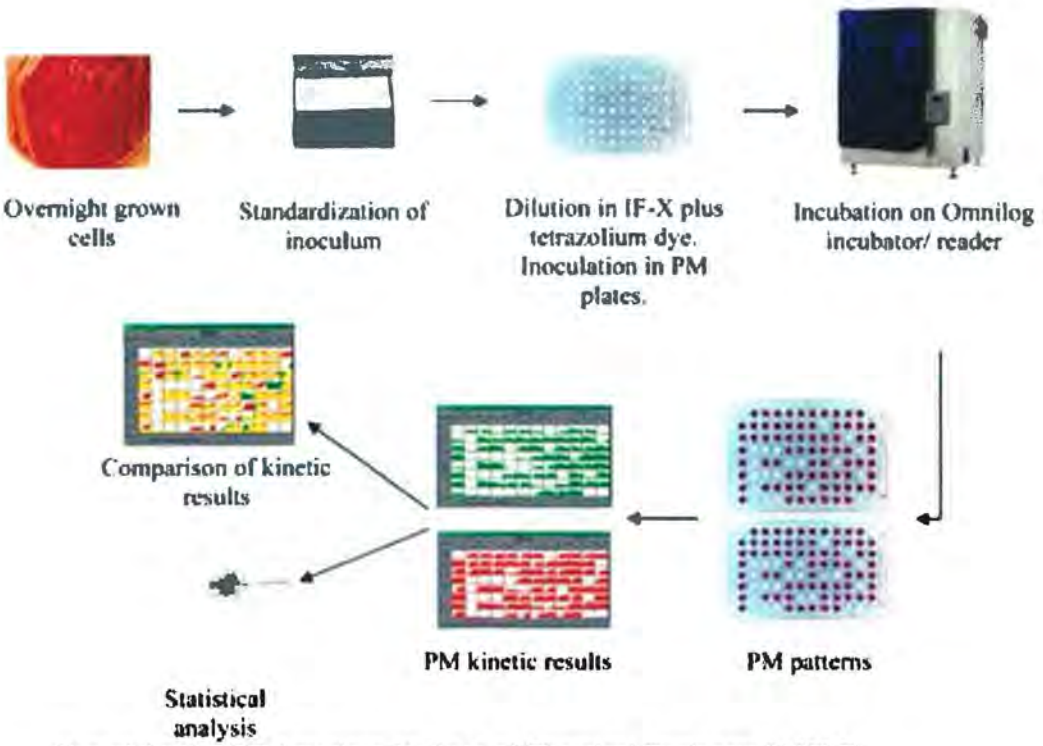


Figure 2.2 Experimental stages of the Phenotype MicroArrays technique

2.8.1 Inoculum preparation

A culture of *Listeria monocytogenes* ATCC 7644, on BUG + B (Biolog Universal Growth medium + Blood) agar with 5% sheep blood, was grown overnight at 37°C and sub-cultured a second time. Using an sterile cotton swab, cells were removed from the plate and transferred into a sterile capped tube containing 20 mL of inoculating fluid (1x IF-0a GN/GP Base, Biolog) to obtain a uniform suspension. Cell density was adjusted to 81% transmittance (T) on a Biolog turbidimeter, equivalent to approx. $1x10^8$ CFU/mL.

2.8.2 Inoculation fluids preparation

PM additive solutions were prepared as 12x stock solutions, filter sterilised and stored at 4°C. Additive solutions differed in their constituents, depending on the PM plate and test assay. Inoculating fluids were prepared at 1x concentration by mixing the appropriate PM additive stock solutions, Biolog Redox Dye Mix F (100x), inoculation fluid, IF-0a GN/GP Base (1.2x) for PM 1 – 8 or IF-10b GN/GP Base (1.2x) for PM 9 – 20, and previously prepared cells. Control plates for all assays were prepared using the *L. monocytogenes* and no test compound. CFA derivatives were added to the inoculation fluids to obtain a final concentration of 0.75 MIC. The appropriate PM inoculating fluid (100 µL) was added to each of the wells. A final cell concentration of approx. $7.3x10^6$ CFU/mL in each well was obtained.

2.8.3 Phenotype MicroArray assay

The PM plates were incubated at 37°C in the OmniLog PM system (incubator and reader) (Biolog, Inc.), and readings were recorded by the charge-coupled device (CCD) camera every 15 min for 24-48 h.

2.8.4 Phenotype MicroArray analysis

Data were analyzed with Omnilog-PM software (Biolog, Inc.), which generated a time course curve for tetrazolium colour formation providing quantitative and kinetic information about the response of cells in the PM plates. The tetrazolium violet used in the system is a redox dye and serves to indicate the utilization of a PM source. Positive utilization reactions are indicated when a purple colour forms in the wells, if there is no reaction, wells stay colourless. Therefore the results were based on the effect in the presence or absence of CFA derivatives on the utilizable vs. non-utilizable response of cells, to each PM source.

Unique phenotypic responses expressed by only one of control or treated cells were identified by a unique colour assigned to that cell line in the kinetic curve. Where there are similar phenotypic responses in both control and treated cell lines, the software can combine both results and a different colour on the kinetic curve is generated (See Appendix 2.2 for kinetic curves obtained). This allows the rapid review of the graphic output and visual identification of changes between control and treated cells.

Biolog facilitates cut-off thresholds to allow easier determination of 'hits' for each plate. The data from the Omnilog-PM software were filtered, using average height as the parameter and then transferred to Excel spreadsheets (Microsoft Corporation). Kinetic curves per well were constructed to evaluate the response of the control to each PM source compared to the treated cells. This allowed identification of trends between CFA derivatives related to the glycoconjugate linkage or anomeric configuration.

Additionally, the PM inhibitors in plates PM11 to 20 were grouped according to their mode of action. The response of *Listeria* cells treated with the CFA derivatives, to each of the PM inhibitors, was allocated a score corresponding to the response of the

control cells without CFA derivative. This was related to whether there was a change (yellow colour in table) or not (blank) and if that change was positive or negative for the sensitivity or resistance of the cells towards the PM inhibitors.

2.8.5 Statistical data analysis

The relationship/association between the CFA derivatives, according to the score obtained with the PM inhibitors' modes of action, was statistically analysed with crosstabs and Chi-square using SPSS software, Version 15.

2.9 ANTIMICROBIAL ACTIVITY OF NATURAL ANTIMICROBIALS (ESSENTIAL OIL COMPONENTS AND NISIN)

2.9.1 Bacterial strains

The antimicrobial activity of natural compounds was studied against *Listeria* strains and spoilage microorganisms shown in Table 2.5.

Table 2.5 Bacterial strains tested.

Bacterial strains
Gram-positive
Lactobacillus sakei ATCC15521
Listeria innocua NCTC 11288
Listeria monocytogenes ATCC 7644
Listeria monocytogenes NCTC 11994
Listeria monocytogenes NCTC 7973
Gram-negative
Escherichia coli ATCC 25922
Pseudomonas fluorescens *
*Pseudomonas putida**

* Strains provided by the Department of Life Sciences, University of Limerick, Ireland.

2.9.2 Test compounds

2.9.2.1 Essential oil components

The essential oil (EO) individual components selected for this study and their chemical properties are indicated in Table 2.6. Their chemical structures are given in Figure 2.3. The EO components were obtained from Sigma-Aldrich, of the highest grade

available. Stock solutions (1M) were prepared in 95% ethanol and then diluted in TSB or MRS broth.

Table 2.6 Essential oil individual components characteristics.

Compound	Formula	Molecular Weight	Density (g/cm^3)	Purity	% in EOs
4-Allylanisole	$C_{10}H_{12}O$	148.21	0.965	95%	26.9% in basil
Camphor	$C_{15}H_{24}$	204.36	0.902	95%	< 22% in sage
Carvacrol	$C_{10}H_{14}O$	150.22	0.976	98%	> 40% in oregano
Caryophyllene	$C_{15}H_{24}$	204.36	0.902	98.5%	3-13% in sage
Citral	$C_{10}H_{16}O$	152.24	0.888	95%	40-90% in lemon balm
Eucalyptol	$C_{10}H_{18}O$	154.25	0.924	96%	40-75% in sage
Linalool	$C_{10}H_{18}O$	154.25	0.861	95%	42.3% in basil
Sabinene	$C_{10}H_{18}O$	154.2	-	98%	Marjoram
Terpinen-4-ol	$C_{10}H_{18}$	154.25	0.933	99%	Marjoram
Thymol	$C_{10}H_{14}O$	150.22	0.965	99.5%	>2% in oregano,thyme

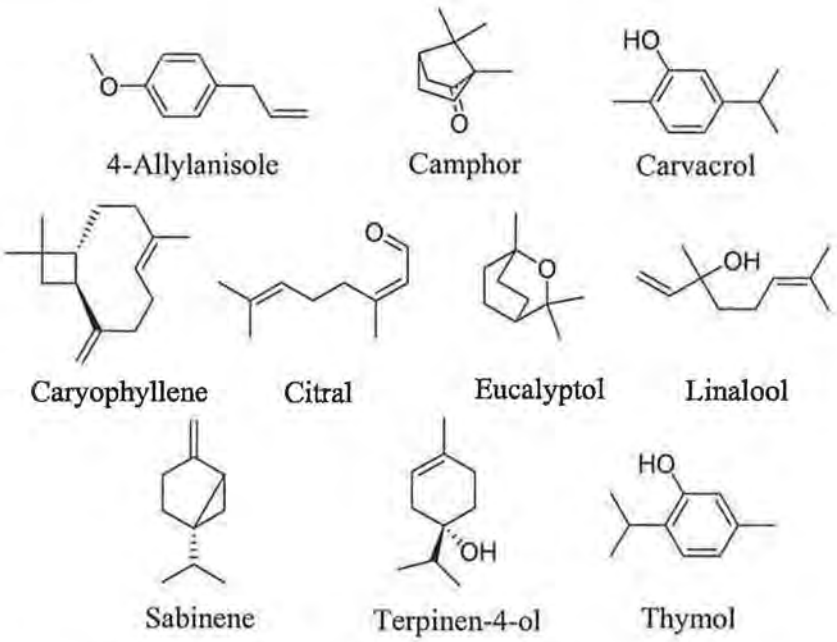
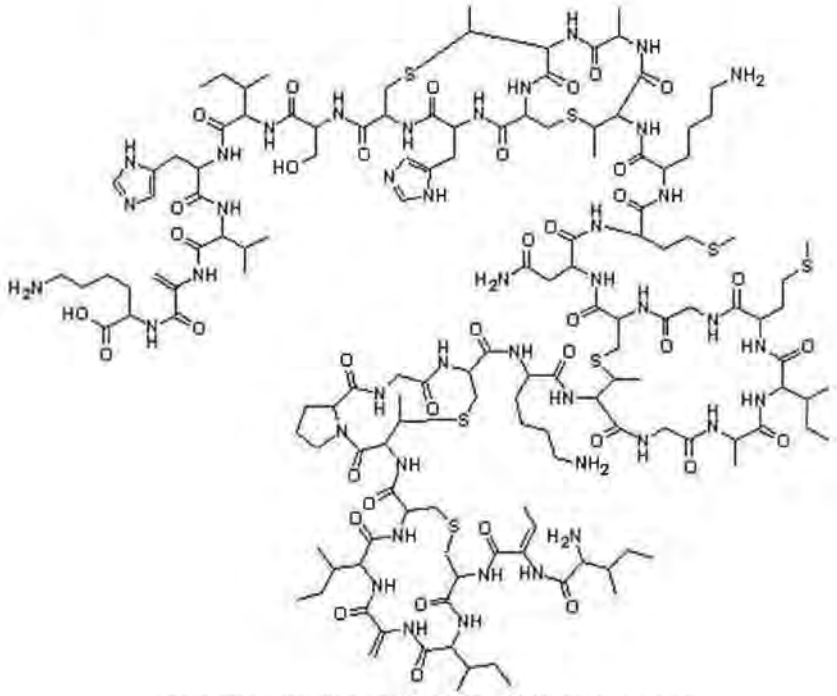


Figure 2.3 Chemical structures of EO components.

2.9.2.2 Bacteriocin

Nisin, from *Lactococcus lactis* 2.5% (≥1.000.000 per IU/g), was purchased from Sigma. Its chemical structure is given in Figure 2.4. A stock solution of 2.5mg/mL was prepared in 0.02N HCl and then further diluted in the appropriate media.



www.chemblink.com/products/1414-45-5.htm

Figure 2.4 Chemical structure of nisin

2.9.3 Absorbance based Microtiter Plate Assay

Ninety-six well microtiter plates (Sarstedt Ltd) were used for the broth microdilution method. This assay was based on previous work (Schelz et al., 2006) but with the following modifications, where aliquots of nisin or EO components solutions in growth media (200 μl) were added into the first row of a microtiter plate. The remainder of the wells was filled with 100 μl of the appropriate medium. The samples were then diluted two fold along each column of the microtiter plate. Concentration ranges used

for the EO components in the antimicrobial assay are given in Table 2.7. Finally, 100 µl of media containing $2x10^6$ CFU/mL of the indicator strain was added to all wells. Positive controls contained only growth media inoculated with the organism under investigation. Negative controls contained nisin or the EO components and sterile growth media only. The plates were then placed in a microplate spectrophotometer (Synergy, Biotek) set at the appropriate temperature. The absorbance was recorded at 600 nm every 30 minutes over a 24 hour incubation period. The Minimum Inhibitory Concentration (MIC) was considered as the lowest concentration of the test sample resulting in a complete inhibition of growth. MIC's of EO individual components were expressed in mM and for nisin in µg/mL. Experiments were performed in duplicate and replicated at least three times.

Table 2.7. Range of concentrations used for the EO components (µg/mL).

mM	4-Allylanisole	Carvacrol, Thymol	Citral	Eucalyptol, Linalool, Sabinene, Terpinen-4-ol	Camphor, Caryophyllene
100	14821	15022	15224	15425	20436
50	7410.5	7511	7612	7712.5	10218
25	3705.25	3755.5	3806	3856.25	5109
20	2964.2	3004.4	3044.8	3085	4087.2
10	1482.1	1502.2	1522.4	1542.5	2043.6
5	741.05	751.1	761.2	771.25	1021.8
2.5	370.53	375.55	380.60	385.63	510.90
1.25	185.26	187.78	190.30	192.81	255.45

2.10 EO COMPONENTS AND NISIN SYNERGY STUDIES

Combinations of selected EO components and nisin were qualitatively assessed. The assay was performed as described in section 2.3.1 but instead of 100µl, 50 µl of the supernatant were added to the EO component solutions into the wells at ratio of 1:1. The potential synergy of the EO components in combination with nisin was quantified by the Fractional Inhibitory Concentration (FIC) indices, which were derived using 96-well microtiter plates. The first row of each plate contained 100 µl of the supernatant, the EO components dilution, or the EO components in combination with nisin (1:1) diluted in TSB or MRS as appropriate.

The initial concentrations for each of the antimicrobials were the MIC's as previously determined. Wells containing the antimicrobials in the first row were then diluted two fold along each column until the end row was reached for each column. Then, 100 µl of media containing $2x10^6$ CFU/mL of the bacterium were added to all wells. At least 2 columns were used for controls. Positive controls contained growth media inoculated with the organism under investigation. Negative controls contained EO constituents or nisin and sterile growth media only. The plates were then placed in the microplate spectrophotometer (Synergy, Biotek) set at the appropriate temperature. Absorbance readings were taken at 600 nm every 30 min after 24 hours of incubation. All assays were performed in duplicate and then replicated.

2.10.1 Determination of the mode of interaction

The MIC data were transformed to fractional inhibitory concentrations (FIC). The FIC of an individual antimicrobial compound is the ratio of the concentration of the

antimicrobial in an inhibitory combination with a second test compound to the concentration of the antimicrobial alone as follows:

$$FIC_A = (MIC_A \text{ combination} / MIC_A \text{ alone})$$

The FIC indices were calculated as follows with the FICs for the individual antimicrobials:

FIC index = $FIC_A + FIC_B$, where FIC_A and FIC_B are the minimum concentrations that inhibited the bacterial growth for samples A and B, respectively. The results were interpreted as synergy (FIC < 0.5), addition (0.5 ≤FIC ≤4) or antagonism (FIC > 4).

2.11 Influence of pH on the effect of bacteriocin and EO

INDIVIDUAL COMPONENTS

The effect of pH on the inhibiting capacity of nisin and/or the EO components was assessed as described above; using the bacterial broths adjusted at pH values 5.5, 6 and 7. The desired pH values were obtained by the addition of 1 M HCl or 1 M NaOH when appropriate.

3. DESIGN, SYNTHESIS AND ANTIMICROBIAL EFFICACY OF CFA DERIVATIVES

This study involved the design, chemical synthesis and antimicrobial evaluation of a series of compounds consisting of a fatty acid component conjugated to a hydrophilic core. The chemical syntheses were developed as part of an iterative design process in response to the antimicrobial activity results, allowing investigation of any structure/activity relationship. The series was designed to investigate the effects of carbohydrate versus non-carbohydrate hydrophilic cores, the glycoconjugate linkage, the length of fatty acid chain, the number of fatty acids attached to the hydrophilic core and the monosaccharide core itself (and the anomeric configuration with respect to glucopyranoside). Fatty acid derivatives were screened for quantitative assessment of antimicrobial activity compared to structurally similar commercial products.

3.1 RESULTS

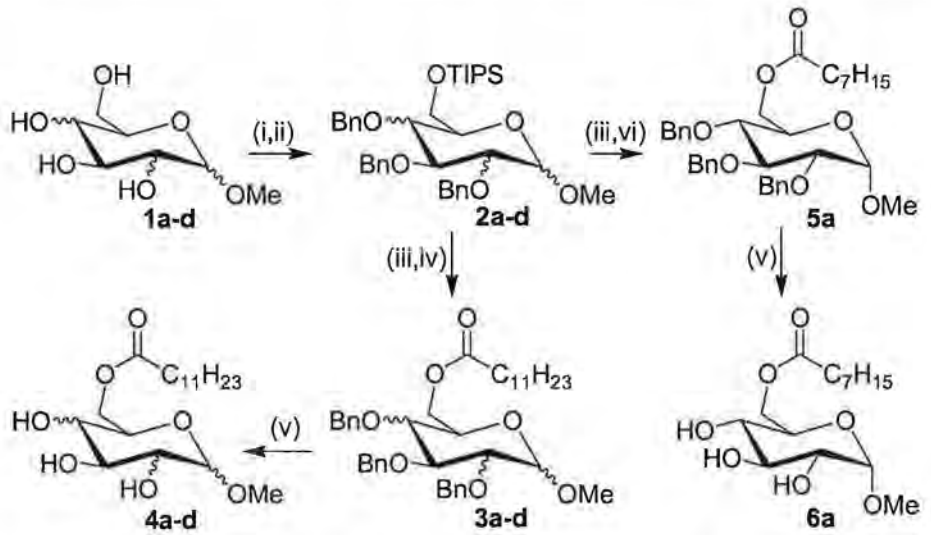
3.1.1 Chemical Synthesis of Carbohydrate Fatty Acid Derivatives

The general synthetic strategy involved a series of protection steps to permit the functionalisation of the primary hydroxyl of the hydrophilic core. Thus, a chemical route has been developed to allow the synthesis of a number of pure, regio-defined, monosaccharide mono-fatty acid esters (Scheme 3.1) and the corresponding ether derivatives (Scheme 3.2). In order to establish whether a second fatty acid conjugated to a monosaccharide would improve antimicrobial activity, a route was developed to synthesise a di-laurate derivative of glucopyranoside (Scheme 3.3). Furthermore, to

investigate whether the structure and, therefore, the synthesis could be simplified and retain activity, non-carbohydrate hydroxylated esters based on a pentaerythritol core were synthesised by a straightforward esterification (Scheme 3.4).

3.1.1.1 General synthesis of ester derivatives

A chemical route designed to obtain mono-ester sugars is shown in **Scheme 3.1** and is based on the following carbohydrate starting materials: methyl α-D-glucopyranoside (**1a**), methyl β-D-glucopyranoside (**1b**), methyl α-D-mannopyranoside (**1c**) and methyl α-D-galactopyranoside (**1d**). The synthesis commenced with the selective protection of the primary hydroxyl of sugars **1a-d** with a triisopropylsilyl (TIPS) group. The silyl derivatives were then fully protected with benzyl groups (Bn) to give **2a-d**. The removal of the TIPS group by tetrabutylammonium fluoride in THF allowed for the esterification of the free 6-OH position with either lauroyl chloride to yield **3a-d** or octanoyl chloride to yield **5a**. Removal of the benzyl groups by catalytic hydrogenation led to the unprotected carbohydrate esters **4a-d** and **6a** respectively. See Appendix 1 (Smith *et al.*, 2008 Carbohydrate Research paper) for the experimental section containing further details of the syntheses, including yields and analytical data for purity and in support of the assignment of the structures.

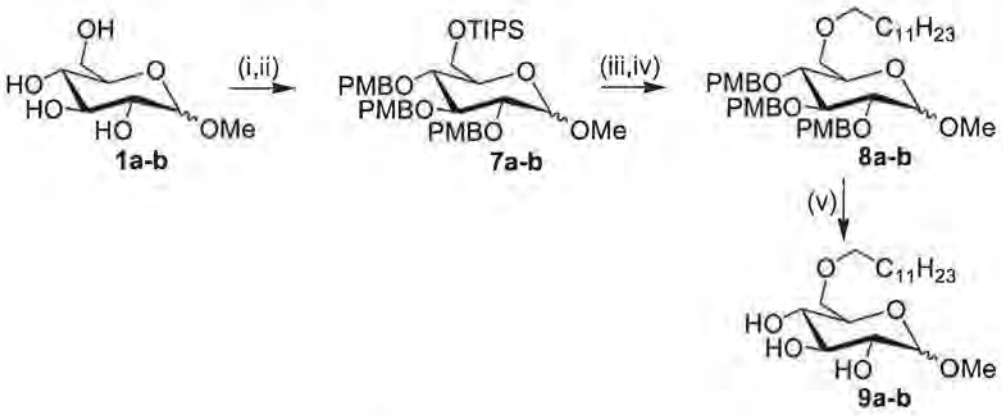


Scheme 3.1. Synthesis of ester derivatives.

Reagents and Conditions: (i) DMF anhydr., TIPSCl, imidazole, rt. (ii) DMF anhydr., NaH, BnBr, rt. (iii) THF anhydr., 0°C, TBAF. (iv) Pyr anhydr., DMAP, Lauroyl Cl, rt. (v) EtOH, Pd-C, H_2. (vi) Pyr anhydr., DMAP, Octanoyl Cl, rt

3.1.1.2 General synthesis of ether derivatives

Synthesis of the ether derivatives also commenced with the protection of the primary hydroxyl with a triisopropylsilyl group **(Scheme 3.2)**. Attempts to use Bn protecting groups for the ether derivatives were unsuccessful as the fatty acid ether was not stable enough to withstand the hydrogenation process which was required for debenzylation. The sugars were instead fully protected using paramethoxybenzyl ethers (PMB), to yield **7a-b**. Removal of the TIPS group gave the free primary hydroxyl. Next, the lauric ether group was attached using dodecanyl chloride to give the fully protected ether derivatives **8a-b**. Finally, oxidative cleavage of the PMB groups with ceric ammonium nitrate (CAN) gave the mono-dodecanyl sugars **9a-b**.

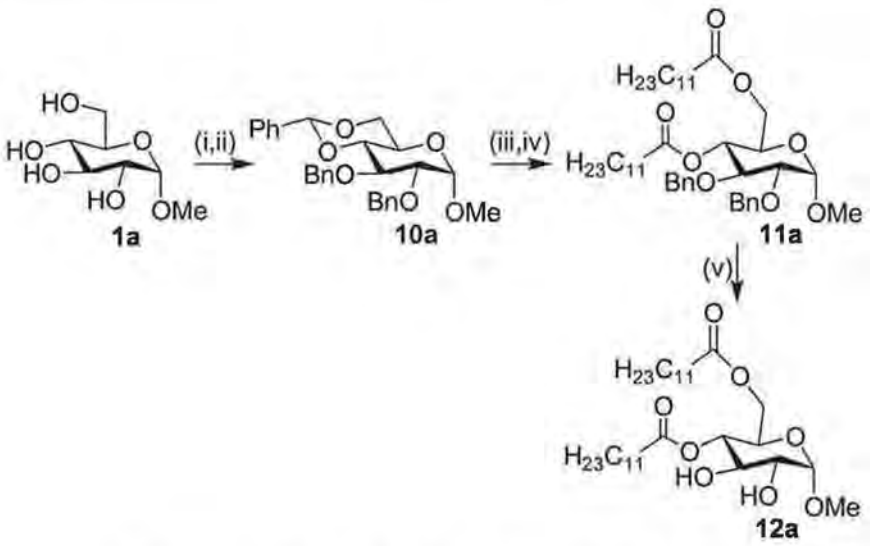


Scheme 3.2. Synthesis of ether derivatives.

Reagents and Conditions: (i) DMF anhydr., TIPSCl, imidazole, rt. (ii) DMF anhydr., THF anhydr., 0°C, NaH, PMBCl, TBAI. (iii) THF anhydr., 0°C, TBAF. (iv) DMF anhydr., dodecanyl chloride, 0°C, NaH. (v) MeCN:H_2O 3:1, CAN, rt.

3.1.1.3 Synthesis of a di-laurate derivative

The method used to synthesise di-lauroyl derivative **12a** is shown in **Scheme 3.3**. The 4 and 6-OH positions of methyl α-D-glucopyranoside **1a** were protected with a benzylidene group using benzaldehyde dimethylacetal. The remaining free OH's were then converted to benzyl ethers to give **10a**. Removal of the benzylidene acetal using catalytic TsOH in MeOH then enabled the esterification of the 4 and 6-OH to give **11a.** Finally, removal of the benzyl groups by catalytic hydrogenation gave the diester derivative **12a**.

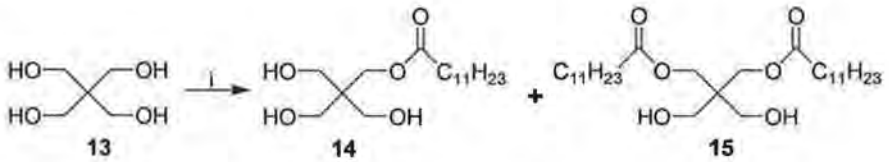


Scheme 3.3. Synthesis of a di-lauroyl derivative.

Reagents and Conditions: (i) TsOH, $PhCH(OMe)_2$, MeCN anhydr., rt. (ii) DMF anhydr., NaH, BnBr, rt. (iii) MeOH, TsOH. (iv) Pyr anhydr., DMAP, Lauroyl Cl, rt. (v) EtOH, Pd/, H_2.

3.1.1.4 Synthesis of non-carbohydrate hydroxylated esters

Direct esterification of pentaerythritol **13** using lauroyl chloride and DMAP in pyridine, yielded the non-sugar derivatives **14** and **15**, shown in **Scheme 3.4**.



Scheme 3.4. Synthesis of non-carbohydrate hydroxylated esters.

Reagents and Conditions: (i) Pyr anhydr., DMAP, Lauroyl Cl, rt.

3.1.2 Bacterial growth and calibration curves

Bacterial growth curves were constructed to determine cell growth and density patterns of each bacterial strain at their optimum growth temperature, to establish the appropriate incubation time needed for the antimicrobial experiments and to compare the effect of the antimicrobial compounds on bacterial growth under optimum conditions.

Figure 3.1 shows example growth curves of selected bacteria used in this study. From the growth curves shown below we can observe that *Listeria* strains (Figure 3.1 **a** and **b**), for example, reached lower OD_{600} values after 18 hours compared to the *S. aureus* strains (Figure 3.1 **c** and **d**). The Gram negative strains, *E. coli* and *P. fluorescens* showed a shorter lag phase compared to the Gram positive strains.

Bacterial calibration curves were constructed to equate absorbance values with cell growth and density measured in Log_{10} cfu/mL (Figure 3.2). Absorbance values were plotted against the Log_{10} cfu/mL to obtain a logarithmic trend line. The coefficient of equations and the R^2 values were obtained, which were subsequently used for the determination of IC_{50} values.

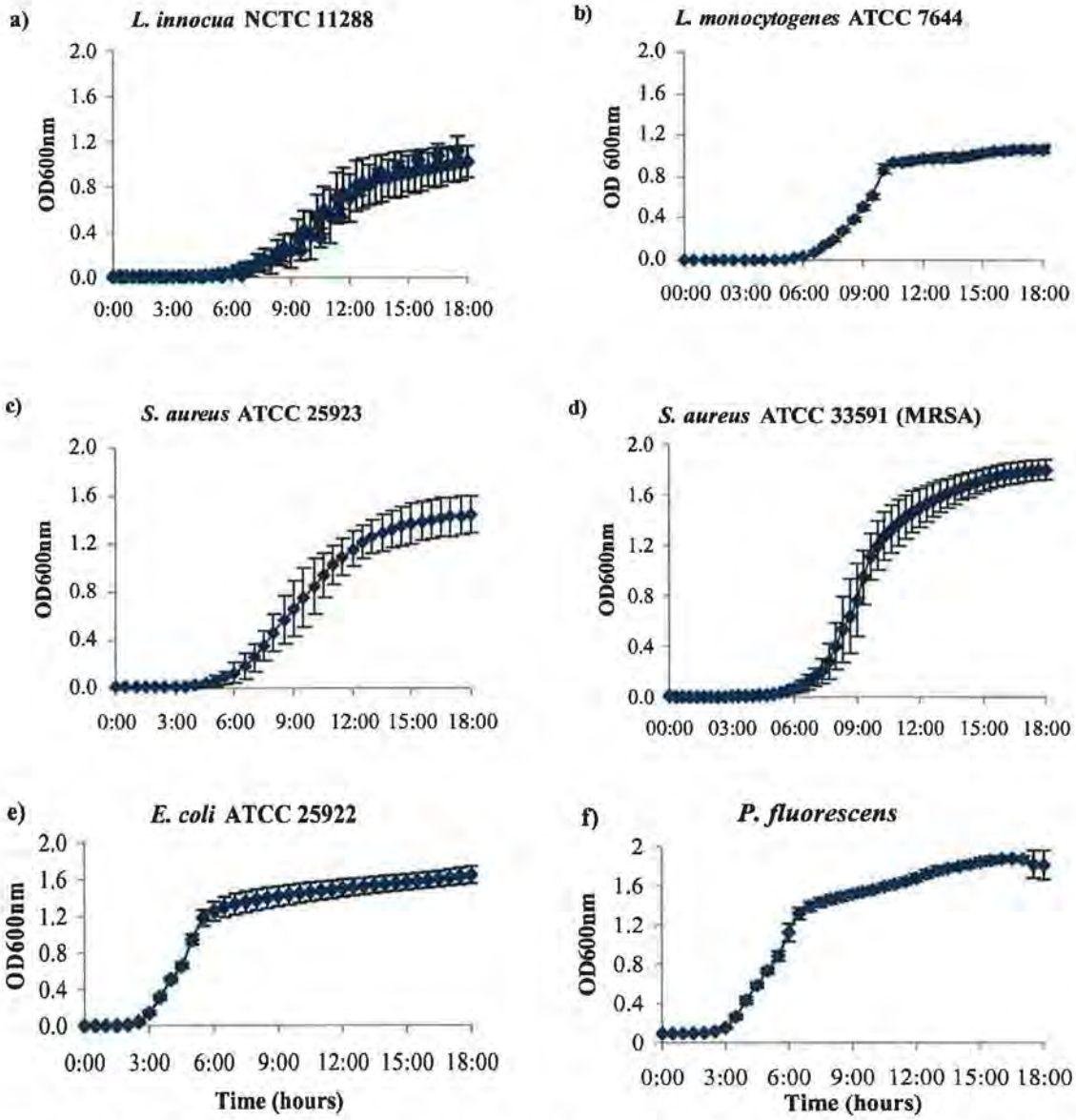


Figure 3.1 Bacterial growth curves in TSB at 35°C. Error bars represent standard deviation values of at least 3 replicates.

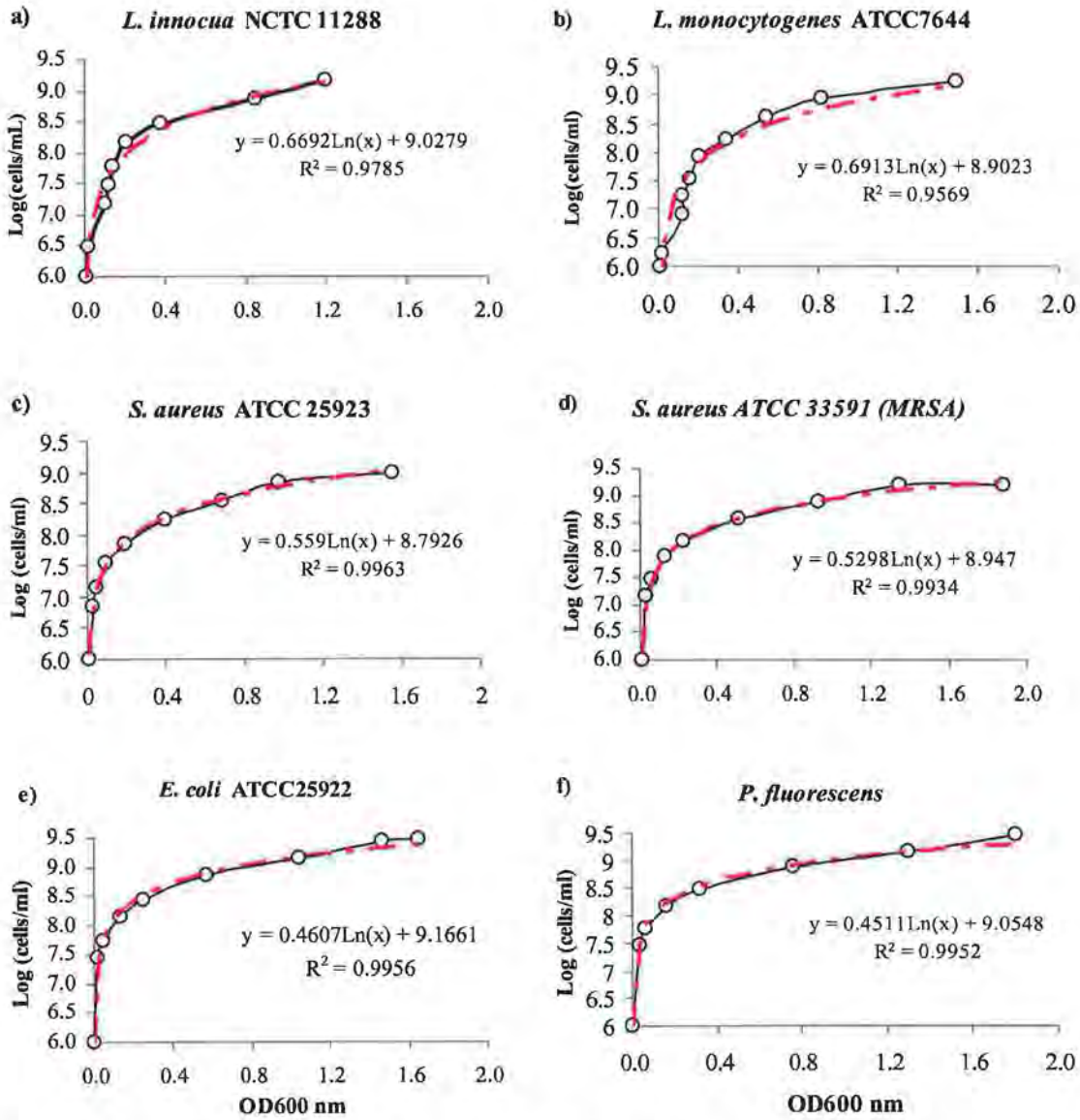


Figure 3.2 Bacterial calibration curves. Trend line in red.

3.1.3 Antimicrobial activity of carbohydrate fatty acid derivatives

Six carbohydrate fatty acid ester derivatives (**4a-d**, **6a**, **12a**), two carbohydrate medium chain alkyl ether derivatives (**9a-b**) and two non-carbohydrate polyhydroxylated fatty acid ester derivatives (**14**, **15**), together with their corresponding polyhydric alcohols (**1a-d**, **13**), fatty acids and monoglycerides as controls, were tested against a range of Gram positive and Gram negative bacteria to determine their antimicrobial activity. The efficacy of the derivatives and controls were compared using MIC, IC_{50} values and increase in Lag phase.

3.1.3.1 Minimum Inhibitory Concentration

Minimum Inhibitory Concentration values (MIC) were defined as the lowest concentration of compound that showed no increase in cell growth for all the replicates compared to a negative control after 18 hours. The minimum inhibitory concentration results are shown in Table 3.1. In each antimicrobial efficacy assay, the corresponding polyhydric alcohols (carbohydrates and pentaerythritol) for the fatty acid derivatives were included, and were shown to have no antimicrobial effect on the microorganisms under investigation. Moreover, there was no growth promoting effect noted for these sugars when assessed in combination with TSB (results not shown). Although the concentrations of ethanol corresponding to that within the assay wells with the highest concentrations of compound used (10 mM for the Gram positive and 20 mM for the Gram negative bacteria) had a minor effect on bacteria viability, there was no antimicrobial effect observed at the concentrations used when incorporated with the compounds at MIC levels.

Table 3.1. Minimum Inhibitory Concentration values of Carbohydrate Fatty Acid derivatives and Controls in TSB at 37°C after 18 h.

Microorganism	FA		MG		Carbohydrate fatty acid derivatives					
	LA	CA	ML	MC	4a	9a	4b	9b	6a	4c
Gram positive bacteria										
Listeria innocua NCTC 11288	0.63	5	0.04	2.5	0.08	0.04	0.08	5	0.63	0.04
Listeria monocytogenes ATCC 7644	0.63	> 5	0.04	5	0.08	0.04	0.08	2.5	2.5	0.04
Listeria monocytogenes NCTC 11994	1.25	> 5	0.04	2.5	0.31	0.04	0.16	> 2.5	1.25	0.04
Listeria monocytogenes NCTC 7973	1.25	5	0.04	2.5	0.08	0.04	0.16	> 2.5	0.31	0.04
Staphylococcus aureus ATCC 25923	0.63	10	0.04	5	0.31	0.04	0.04	2.5	2.5	0.04
Staphylococcus aureus NCTC 1803	0.63	10	0.04	2.5	0.31	0.04	0.04	1.25	1.25	0.04
Staphylococcus aureus ATCC 33591 (MRSA)	1.25	10	0.04	2.5	1.25	0.04	0.04	2.5	5	0.04
Staphylococcus aureus ATCC 33592 (MRSA)	1.25	20	0.04	2.5	0.08	0.08	0.04	5	2.5	0.16
Staphylococcus aureus ATCC 43300 (MRSA)	1.25	10	0.08	2.5	0.08	0.08	0.04	10	2.5	0.08
Gram negative bacteria										
Escherichia coli ATCC 25922	> 20	10	20	5	20	20	20	20	12.5	≥20
Escherichia coli NCTC 12900	12.5	10	12.5	5	12.5	10	12.5	10	12.5	N.D
Salmonella Typhimurium ATCC 14028	> 20	> 20	20	> 20	20	> 20	> 20	20	> 20	N.D
Enterobacter aerogenes ATCC 13048	> 20	20	20	10	20	> 20	> 20	> 20	> 20	N.D
Pseudomonas fluorescens	> 20	5	20	5	> 20	> 20	> 20	> 20	5	N.D

For each analysis the MIC was recorded as the concentration (mM) that resulted in total inhibition of all replicates. N.D: Not determined;

FA: Fatty acids; LA: Lauric acid; CA: Caprylic acid; MG: Monoglycerides; ML: Monolaurin; MC: Monocaprylin.

4a. Methyl 6-O-lauroyl-α-D-glucopyranoside; 9a. Methyl 6-O-dodecanyl-α-D-glucopyranoside; 4b. Methyl 6-O-lauroyl-β-D-glucopyranoside; 9b. Methyl 6-O-dodecanyl-β-D-glucopyranoside; 6a. Methyl 6-O-octanoyl-α-D-glucopyranoside; 4c. Methyl 6-O-lauroyl-α-D-mannopyranoside

Examples of survivor curves for *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, obtained using kinetic absorbance assays, are also presented in Figure 3.3 and Figure 3.4 respectively.

As can be observed in Table 3.1, the monoglycerides (ML, MC) had greater activity ($p<0.05$) against Gram positive microorganisms than the free fatty acids (LA, CA), and comparable activity at the concentrations tested against Gram negative microorganisms. Of the monoglycerides and free fatty acids tested, ML had the lowest MIC values ($p<0.05$) and was particularly effective for inhibition of Gram positive microorganisms with MIC values of 0.04 mM (excluding *S. aureus* ATCC43300 with an MIC of 0.08 mM), by comparison with the range observed for LA which had MIC values between 0.63 mM to 1.25 mM.

A similar trend was observed for MC when compared with the free fatty acid CA. Minimum inhibitory concentration values between 2.5 mM and 5 mM were recorded for MC against the Gram positive bacteria tested, in contrast to CA, which had MIC value $\geq$ 5 mM, being the least effective of all compounds tested against Gram positive bacteria with MICs 8- to 16-fold lower than those of lauric acid.

When tested against the Gram negative bacteria, LA and ML had no activity at concentrations up to 20 mM (Table 3.1). An exception to this was recorded for *E. coli* NCTC12900 with a MIC value of 12.5 mM for LA and ML. *P. fluorescens* was susceptible to CA and MC at a concentration of 5 mM for both compounds, whereas for *E. coli* strains (ATCC 25922 and NCTC 12900), MIC values were 10 mM and 5 mM respectively. Minimum inhibitory concentrations of CA were $\geq$20 mM for the other Gram negative bacteria (Table 3.1).

All CFA derivatives tested showed greater antimicrobial activity against Gram positive microorganisms than Gram negative ($p<0.05$). For Gram positive bacteria, the lauric ether of alpha glucopyranoside (compound **9a**) and lauric ester of alpha mannopyranoside (compound **4c**) were the most active derivatives with a MIC range of 0.04 mM to 0.08 mM, comparable to ML (Table 3.1). The next compound in order of overall efficacy was the beta lauric ester of glucopyranoside (compound **4b**), with MIC values of 0.04 mM for *S. aureus* strains and between 0.08 mM and 0.16 mM for *Listeria* strains. This was followed by the lauric ester of alpha glucopyranoside (compound **4a**) with a greater MIC range of 0.08 mM to 1.25 mM. The antimicrobial activity of lauric ether of beta glucopyranoside (compound **9b**) with MIC values from 1.25 mM up to 10 mM was significantly lower ($p<0.05$) than that observed with the corresponding lauric ether of alpha glucopyranoside (**9a**).

The caprylic ester of alpha glucopyranoside (compound **6a**) had a greater activity ($p<0.05$) compared to MC against *Listeria* strains, with MIC values between 0.31 mM and 2.5 mM. For *S. aureus* strains, the activity of alpha caprylic ester was comparable to that of MC, with MIC values of 1.25 mM to 5 mM (Table 3.1). Compound **6a** was more active than the lauric acid derivatives (**4a-c**, **9a-b**) against *E. coli* ATCC 25922 and *P. fluorescens*, with MIC values of 12.5 mM and 5 mM respectively (Table 3.1).

Compound **14** (a non-carbohydrate mono-ester) was evaluated, but its antimicrobial activity was negligible (results not shown). Compounds **4d**, **12a** and **15** could not be accurately tested for antimicrobial efficacy due to poor solubility in water.

The results presented in Figure 3.3 show the survivor curves of *S. aureus* ATCC 25923 as an example of a Gram positive organism, at a range of concentrations (shown on left hand column) for the controls and synthesized compounds tested.

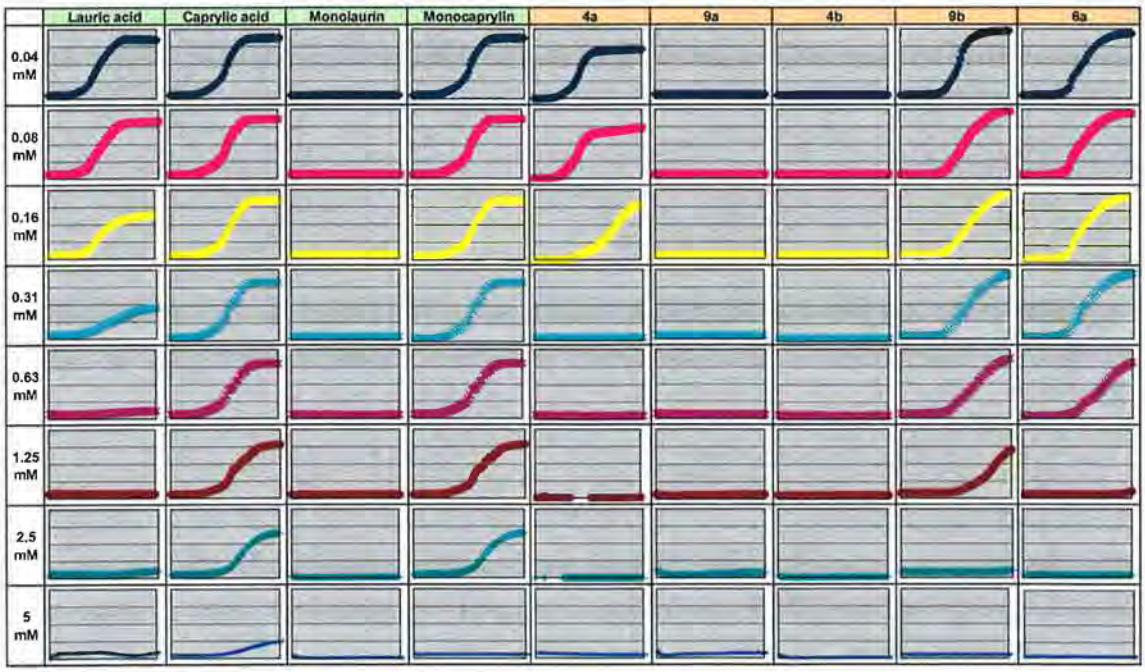


Figure 3.3: Antimicrobial effect of CFA derivatives against *S. aureus* ATCC 25923. The vertical axis in all plots represents OD_{600} while the horizontal axis is time (18 hours). **4a.**) Methyl 6-O-lauroyl-α-D-glucopyranoside; **9a.**) Methyl 6-O-dodecanyl-α-D-glucopyranoside; **4b.**) Methyl 6-O-lauroyl-β-D-glucopyranoside; **9b.**) Methyl 6-O-dodecanyl-β-D-glucopyranoside; **6a.**) Methyl 6-O-octanoyl-α-D-glucopyranoside.

It is clear from the comparison of survivor curves within Figure 3.3, that the most effective control compound tested was ML (MIC: 0.04 mM); with comparable efficacy noted for alpha lauric ether (compound **9a**) and beta lauric ester (compound **4b**) of the CFA derivatives (MIC: 0.04 mM). The antimicrobial efficacy of the compounds was found to be concentration dependent.

Figure 3.4 shows the survivor curves of *E. coli* ATCC 25922 as an example of Gram negative organism at a range of concentrations (shown on the left hand column) for the controls and synthesized compounds tested.

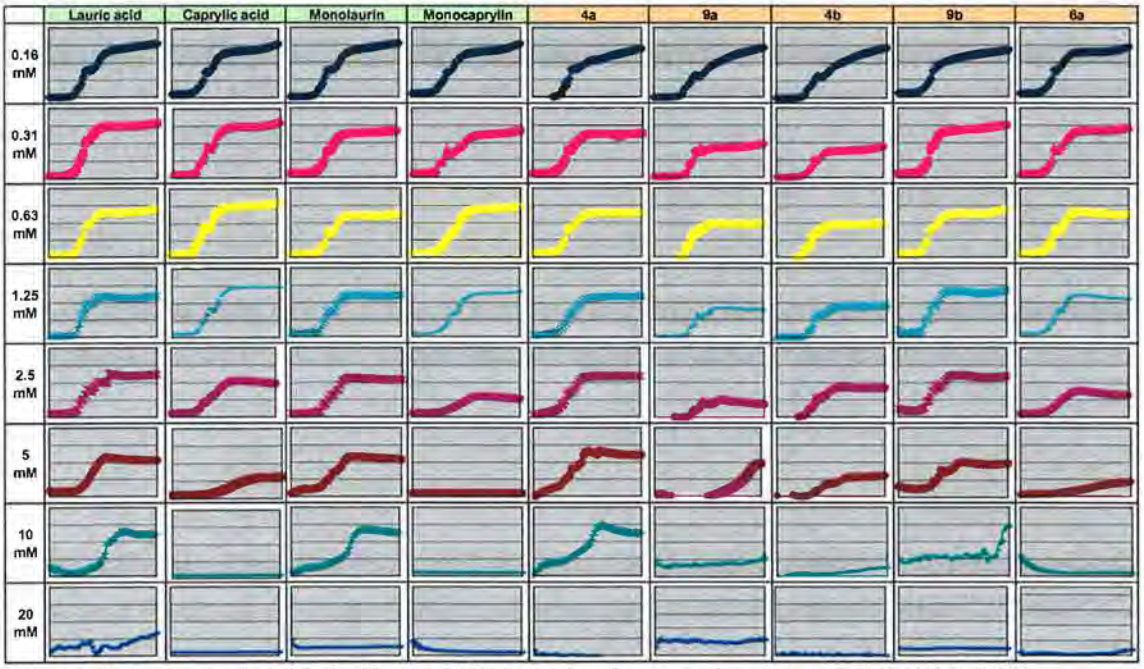


Figure 3.4: Antimicrobial effect of CFA derivatives against *E. coli* ATCC 25922.
The vertical axis in all plots represents OD_{600} while the horizontal axis is time (18 hours).
4a.) Methyl 6-O-lauroyl-α-D-glucopyranoside; **9a.**) Methyl 6-O-dodecanyl-α-D-glucopyranoside; **4b.**) Methyl 6-O-lauroyl-β-D-glucopyranoside; **9b.**) Methyl 6-O-dodecanyl-β-D-glucopyranoside; **6a.**) Methyl 6-O-octanoyl-α-D-glucopyranoside.

As can be seen from Figure 3.4, MC (5 mM) and CA (10 mM) had a greater activity against *E. coli* ATCC 25922 at lower concentrations than either LA or ML controls at equivalent concentrations. The efficacy of the control and synthesized compounds was concentration dependent. The carbohydrate fatty acid derivatives showed minor activity only at the higher concentration tested (20 mM). An exception was for the caprylic ester of alpha glucopyranoside (**6a**) with antimicrobial activity observed at 12.5 mM.

3.1.3.2 IC_{50} values

The MIC values reported above (Table 3.1) were based on growth or non-growth data. An example of the dose-response curve for *Listeria monocytogenes* ATCC 7644 with different concentrations of lauric acid is shown in Figure 3.5.

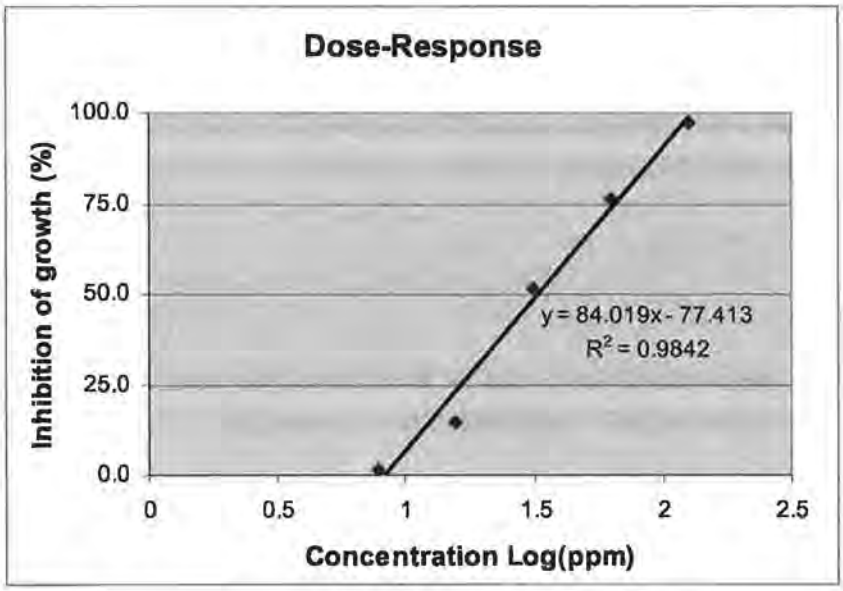


Figure 3.5: Dose response curve of *Listeria monocytogenes* ATCC 7644 with different concentrations of lauric acid.

Experiments were performed to evaluate the extent to which sub-MIC concentrations influenced bacterial growth. IC_{50} values were determined for the fatty acids, lauric and caprylic acid, beta lauric ether (**9b**) and alpha caprylic ester (**6a**), and are summarized in Table 3.2

When the IC_{50} values were compared, some trends similar to those recorded for the MICs were observed. For example, lauric acid was again more effective against all bacteria than the caprylic acid. Differences between strains were also observed, for example, *L. innocua* NCTC 11288, a surrogate of *L. monocytogenes* but non-pathogenic

in character, was more resistant for the beta lauric ether (**9b**) with an IC_{50} value of 0.879 mM compared to *L. monocytogenes* ATCC 7644 with an IC_{50} value of 0.384 mM. the same profile was observed for the *S. aureus* strains, with IC50 values of 0.587 mM and 0.904 mM for *S. aureus* ATCC 25923 and *MRSA* ATCC 33591, respectively, with the MRSA strain being more resistant. Similar trends were observed for the alpha caprylic ester (**6a**) and *S .aureus* strains, with IC_{50} values of 0.700 mM and 1.090 mM. On the other hand, the MRSA strain was shown to be more susceptible than the *S. aureus* to caprylic acid with IC_{50} values of 2.749 mM and 5.111 mM correspondingly (Table 3.2).

Table 3.2: IC_{50} values of fatty acids and carbohydrate fatty acid derivatives.

Microorganism	Lauric acid	Beta lauric ether (9b)	Caprylic acid	Alpha caprylic ester (6a)
***L. innocua* NCTC 11288**	0.168	0.879	N.D	N.D
***L. monocytogenes* ATCC 7644**	0.164	0.384	4.842	0.441
***S. aureus* ATCC 25923**	0.288	0.587	5.111	0.700
***MRSA* ATCC 33591**	0.214	0.904	2.749	1.090

Values are expressed as concentration (mM). N.D: not determined.

3.1.3.3 Increase in lag time and decrease in maximum specific growth rate

Where appropriate, the increase in lag time was calculated. For the concentrations at which a compound was less effective, an increase in the lag time could be estimated to allow comparison between compounds and strains.

The increase in lag time ($\Delta\lambda$) and decrease in maximum specific growth rate (μ_{max}) were estimated for *L. monocytogenes* ATCC 7644 (Table 3.3) and *S. aureus* ATCC 25923 (Table 3.4) to allow further comparison between compound efficacies.

Results were found to be concentration and compound dependent ($p < 0.05$). Generally, the increase in lag time between concentrations of a compound was observed to be more marked than the decrease in growth rate which was more gradual. For example, for *L. monocytogenes* ATCC 7644 at sub-MIC concentrations, compound **4b** (beta lauric ester) had an increase in lag time from 0.5h to 5.3h associated with a small increase in concentration from 0.02 mM to 0.04 mM. This trend was also true for LA, CA, MC and compound **9b** (beta lauric ether) (Table 3.3). With respect to μ-max, different patterns were observed, there was a gradual decrease noted with LA, CA, MC and compound **9b**, associated with the higher MIC values for these compounds. Whereas, for ML and compound **4b**, there was a non-linear association of μ-max reduction with concentration, associated with the very low MIC values determined for these compounds.

For *S. aureus* ATCC 25923, increase in lag time was found to be concentration and compound dependent, with a major effect at concentrations close to the MIC values (Table 3.4) ($p < 0.05$). For example, at 2-fold sub-MIC concentrations, the fatty acids (LA, CA) and monoglyceride (ML, MC) standards, as well as compound **4a** (alpha lauric ester) and compound **6a** (alpha caprylic ester), 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 **9b** (beta lauric ether), a different trend was observed, 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 mM) (Table 3.4).

Table 3.3 Effect of FA, MG and CFA derivatives on the Lag time (λ) and Maximum specific growth rate (μ_{max}) of *L. monocytogenes* ATCC 7644

Compound	(mM)	Δλ (h)	St.Dev.	μ_{max} (h^{-1})	St.Dev.
LA	0	-		0.30	± 0.034
	0.04	0.0	± 0.06	0.22	± 0.049
	0.08	0.2	± 0.26	0.17	± 0.041
	0.16	2.0	± 1.00	0.10	± 0.017
	0.31	4.8	± 1.73	0.07	± 0.037
	0.63	no growth		0	
ML	0	-		0.30	± 0.034
	0.02	2.3	± 1.09	0.25	± 0.040
	0.04	no growth		0	
4a	0.08	no growth		0	
9a	0.04	no growth		0	
4b	0	-		0.30	± 0.034
	0.02	0.5	± 0.07	0.31	± 0.003
	0.04	5.3	± 0.67	0.27	± 0.006
	0.08	no growth		0	
9b	0	-		0.30	± 0.034
	0.16	0.2	± 0.18	0.30	± 0.013
	0.31	0.5	± 0.25	0.27	± 0.009
	0.63	5.0	± 0.55	0.12	± 0.059
	1.25	no growth		0	
CA	0	-		0.30	± 0.034
	0.31	0		0.26	± 0.027
	0.63	0	± 0.04	0.24	± 0.037
	1.25	0.1	± 0.17	0.26	± 0.044
	2.5	0.8	± 0.19	0.21	± 0.034
	5	3.1	± 1.62	0.18	± 0.097
	10	no growth		0	
MC	0	-		0.30	± 0.034
	0.31	0.2	± 0.29	0.26	± 0.029
	0.63	0.3	± 0.40	0.25	± 0.043
	1.25	1.1	± 0.41	0.19	± 0.046
	2.5	5.6	± 1.35	0.01	± 0.034
	5	no growth		0	
6a	0	-		0.30	± 0.034
	0.31	0.4	± 0.47	0.24	± 0.035
	0.63	1.6	± 0.94	0.22	± 0.008
	1.25	1.0	± 0.27	0.12	± 0.008
	2.5	1.9	± 0.34	0.08	± 0.001
	5	no growth		0	

Table 3.4 Increase in Lag time (Δλ) for *S. aureus* ATCC 25923 in the presence of standards and CFA derivatives

Compound	(mM)	Δλ (h)	SD	Compound	(mM)	Δλ (h)	SD
LA	0			**CA**	0		
	0.04	0.0	± 0.000		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			**MC**	0		
	0.02	3.9	± 0.684		1.25	0.0	± 0.000
	0.04	N.G			2.5	1.8	± 0.986
					5	N.G	
4a	0			**6a**	0		
	0.04	0.0	± 0.000		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					
9b	0						
	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; 4a. Methyl 6-O-lauroyl-α-D-glucopyranoside; 9b. Methyl 6-O-dodecanyl-β-D-glucopyranoside; 6a. Methyl 6-O-octanoyl-α-D-glucopyranoside.

3.1.4 Effect of pH and Temperature on the fatty acids and monoglycerides activity

To assess the effect of pH and temperature on the antimicrobial efficacy of the fatty acid derivatives, the growth of selected bacterial strains (*L. innocua* ATCC33090, *S. aureus* ATCC25923, *E. coli* ATCC 25922 and *Pseudomonas fluorescens*) was recorded at different temperatures (35°C, 22°C, 10°C and 4°C) in combination with a range of pHs (4, 4.5, 5, 5.5, 6, 7) in the absence or presence of test compounds (LA or ML).

3.1.4.1 Effect of pH levels on antimicrobial efficacy

The changes in pH values measured after autoclaving the media with adjusted pH levels were minor and deemed to have no major influence on the experiment. Changes in pH, after addition of the compounds, were also recorded to assess possible influence of the addition of compounds in the media on bacterial growth; and were also found to be minor (Table 3.5).

Table 3.5. Effect of fatty acids and monoglycerides on the pH of the culture media.

Media	pH
TSB	7.16 ± 0.007
TSB (10% EtOH)	7.28 ± 0.012
TSB + Lauric acid 5 mM (10% EtOH)	7.10 ±0.007
TSB + Monolaurin 5 mM (10% EtOH)	7.28 ±0.005
TSB + Caprylic acid 5 mM (10% EtOH)	6.93 ± 0.007

Bacterial growth curves were constructed from the OD_{600} data at an optimum temperature of 35°C with the different pH concentrations, in the absence of test compounds (Figure 3.6).

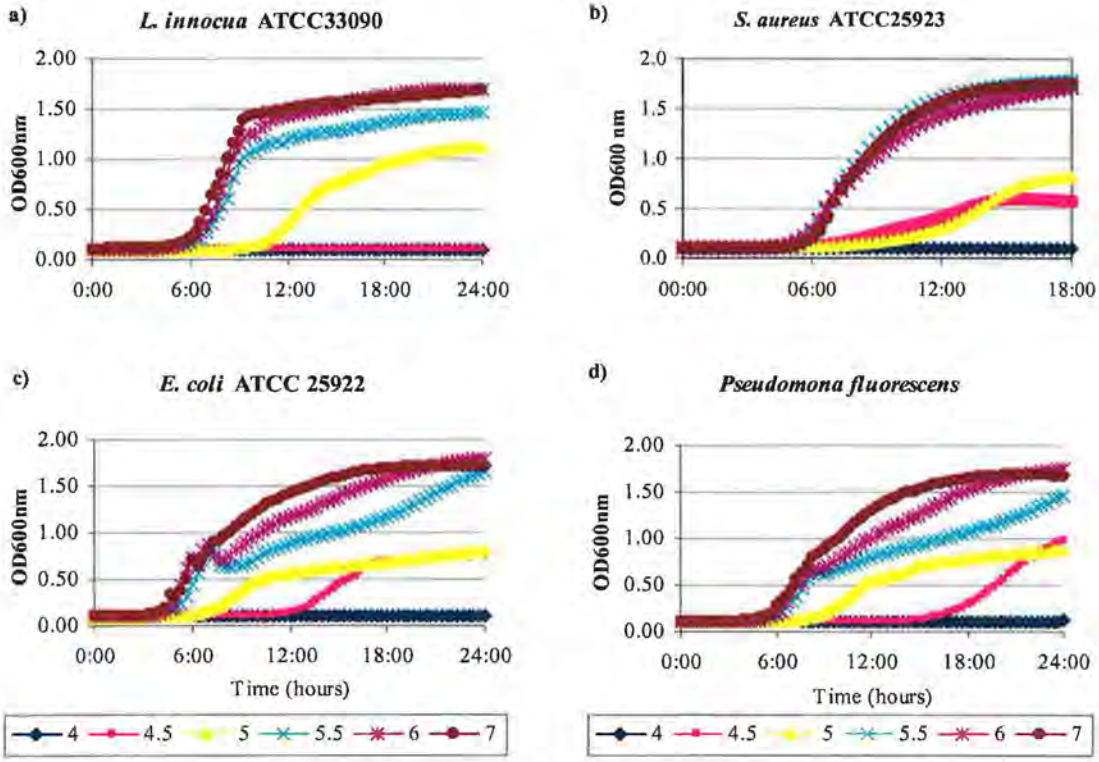


Figure 3.6 Effect of different pH levels on bacterial growth at 35°C in TSB.

After the incubation time, no growth was observed for any of the strains tested at pH 4 in the absence of test compound; therefore further studies did not include pH 4. *L. innocua* ATCC33090 was very susceptible at pH 4.5 with no increase in OD_{600} recorded after 24 hours of incubation; *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. fluorescens* grew at pH 4.5 but had an increased lag time and reduced maximum specific growth rate. In general, results show that as pH lowered, a decrease in the optical density and an increase in the lag phase were observed for each of the strains. For example, the

lag phase of *S. aureus* ATCC 25923 at pH 7 was approximately 5.8 hours, but at lower pH levels the lag phase increased up to 8 hours and 10.5 hours for pH 5 and 4.5, respectively (Table 3.6).

Table 3.6 Effect of pHs on the Lag time (λ)

pH	*L. innocua* ATCC33090	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. fluorescens*
4	N.G	N.G	N.G	N.G
4.5	N.G	N.D	15.5 ± 4.98	17.3 ± 1.35
5	10.8 ± 0.13	10.5 ± 0.05	6.9 ± 0.10	8.8 ± 0.15
5.5	7.6 ± 0.54	5.9 ± 1.28	7.4 ± 1.98	7.5 ± 2.16
6	7.1 ± 0.38	5.7 ± 0.27	5.1 ± 0.42	6.2 ± 0.17
7	6.7 ± 0.23	5.8 ± 0.56	5.1 ± 0.15	5.6 ± 0.12

N.G: No growth. N.D: Not determined. Values are expressed as time in hours. Standard deviations next to each value.

For *Listeria innocua* ATCC33090, a less pronounced increase in the lag phase between pH 7 and 5.5 was observed, but at pH 5 a lag phase of approximately 11 hours was recorded, compared with 6.7 hours at pH 7. A similar trend was observed for the Gram negative bacteria tested, which could grow slowly at pH 4.5 in the absence of test compound: the lag phase increased to 15-17 hours at pH 4.5 compared to 5.6 hours at pH 7.

The effect on the maximum specific growth rate was also affected by pH level. Between pH 4 and 5.5 a marked decrease in growth rate was observed with almost half of the values found at pH 7 (Table 3.7).

Table 3.7 Effect of pH on the maximum specific growth rate (μ_{max})

pH	*L. innocua* ATCC33090	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. fluorescens*
4	N.G	N.G	N.G	N.G
4.5	N.G	N.D	0.124 ± 0.020	0.140 ± 0.013
5	0.174 ± 0.046	0.101 ± 0.003	0.115 ± 0.003	0.124 ± 0.005
5.5	0.325 ± 0.050	0.208 ± 0.013	0.249 ± 0.086	0.186 ± 0.046
6	0.376 ± 0.031	0.252 ± 0.031	0.280 ± 0.025	0.207 ± 0.051
7	0.384 ± 0.026	0.253 ± 0.038	0.232 ± 0.026	0.222 ± 0.005

N.G: No growth. N.D: Not determined. Values expressed as (h^{-1}).
Standard deviations next to each value.

With the addition of antimicrobial compounds at 35°C, there were interactions observed between pH level and compound concentration. No growth was observed for *S. aureus* ATCC 25923 at the range of LA concentrations tested, but at pH 7 (Figure 3.7.a) growth was permitted in the presence of LA at ½ MIC; whereas at pH 5, the efficacy of the compounds was enhanced (Figure 3.7.b), potentially reducing MIC values and concentrations required for useful efficacy.

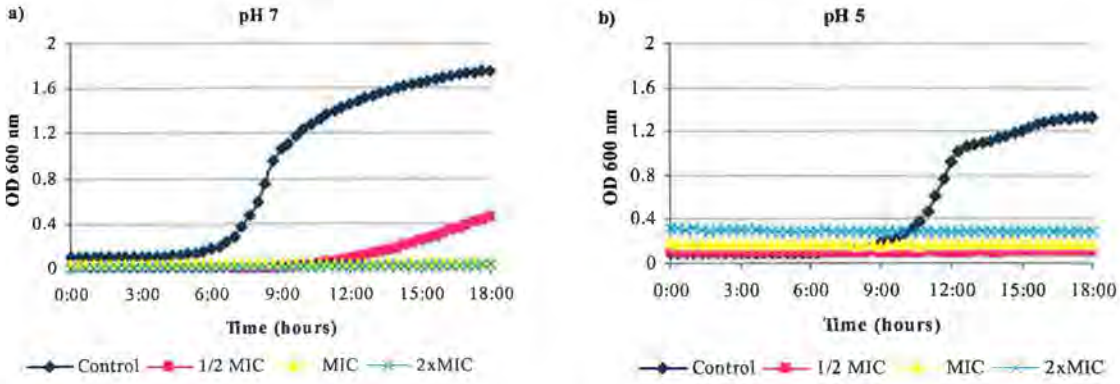


Figure 3.7 Effect of pH on the MIC values of lauric acid against *S. aureus* ATCC 25923 at 35°C.

As a result, the inhibition of growth by lauric acid is dependent not only on concentration but also on pH. Similar findings were obtained by Sun *et al.* (2003) who reported a dramatic effect of pH on the potency of fatty acids.

By contrast, the antimicrobial efficacy of monolaurin was not influenced by pH. Growth at sub-MIC concentrations was observed both at pH 7.0 and pH 5.0 (Figure 3.8).

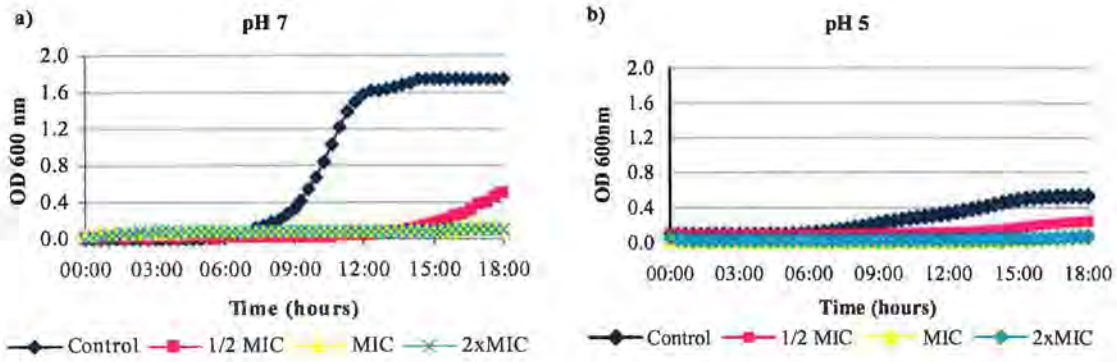


Figure 3.8 Effect of pH on the efficacy of monolaurin against *S. aureus* ATCC 25923 at 35°C.

3.1.4.2 Effect of temperature and pH levels on antimicrobial efficacy

L. innocua ATCC33090 grew at 22°C at all pH levels tested (Figure 3.9.a). The addition of lauric acid at a sub-MIC concentration caused a substantial decrease in growth at all pH levels after 24 hours of incubation (Figure 3.9.b) while growth was completely arrested at MIC concentrations. Similar trends were observed with monolaurin at sub-MIC, however, at 48 hours, higher OD values were achieved by comparison with lauric acid (Figure 3.9.c).

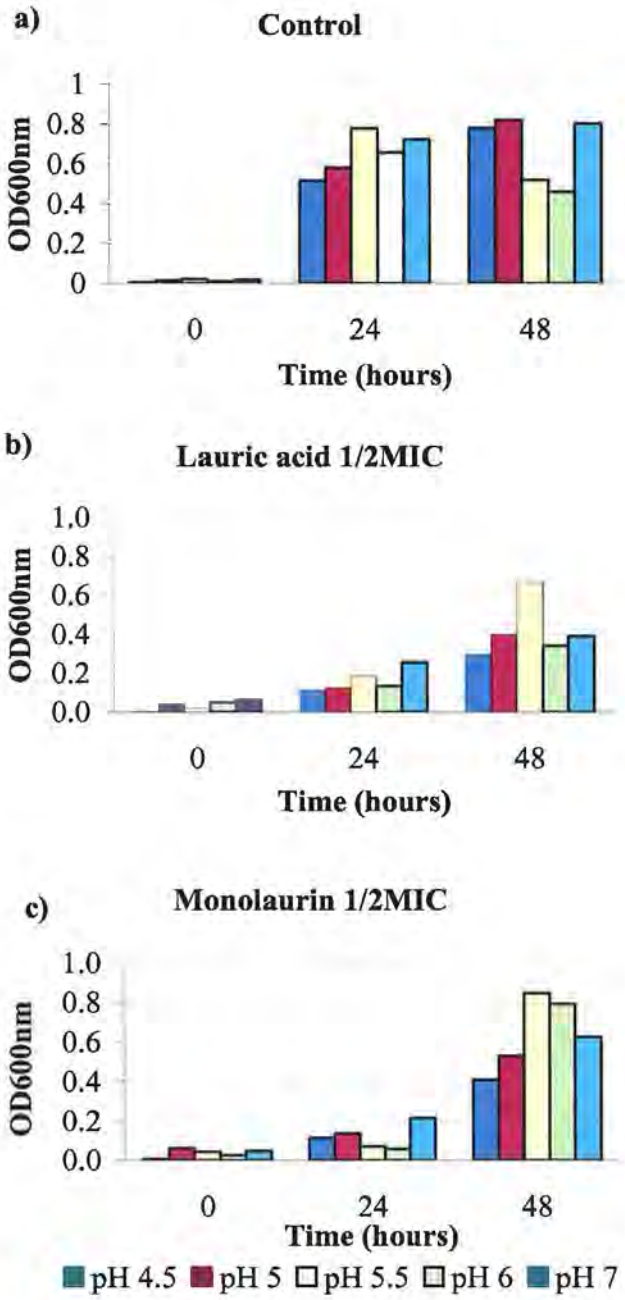


Figure 3.9 Effect of lauric acid and monolaurin at sub-MIC concentrations on the growth of *L. innocua* ATCC33090 at 22°C for different pH levels.

At lower temperatures of 10°C and 4°C, no growth was observed for *L. innocua* ATCC33090, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 after 96 hours even in the absence of antimicrobial compounds. However, *P. fluorescens* grew at 10°C after 96 hours for control samples and those with lauric acid at 1/2 MIC concentration (Figure 3.10).

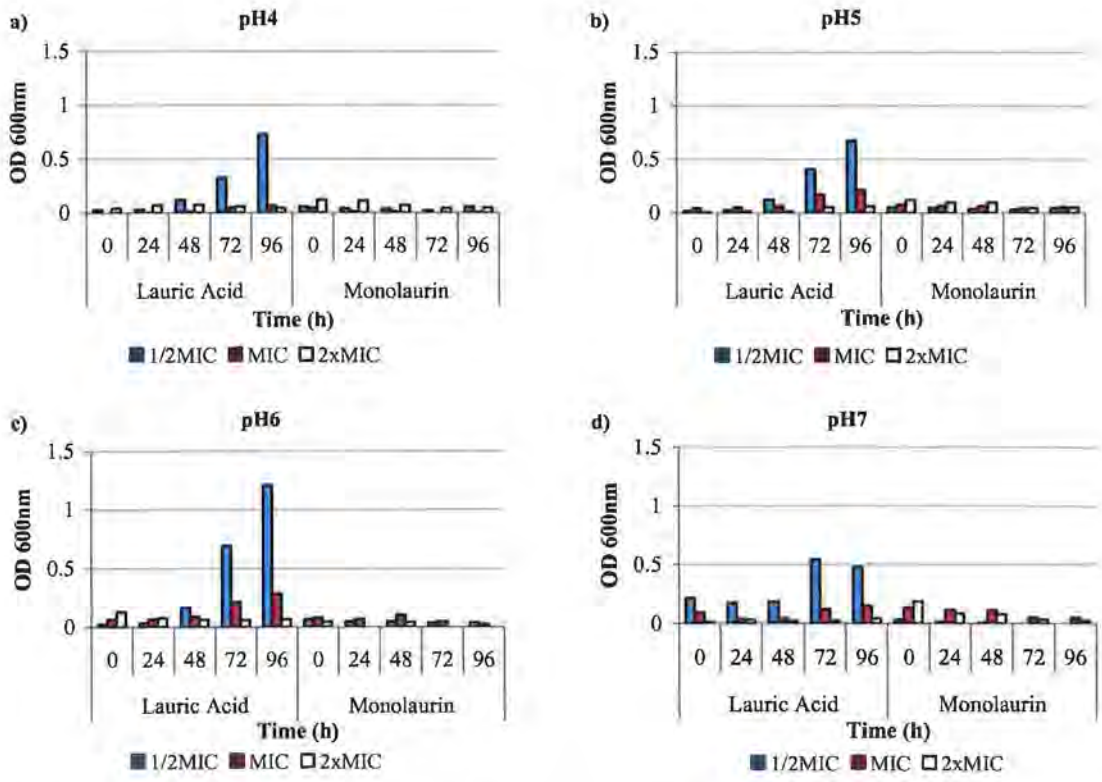


Figure 3.10: Effect of lauric acid and monolaurin concentrations on the growth of *P. fluorescens* at 10°C at different pHs.

As an overall result we observed that pH had an effect on bacterial growth and on antimicrobial efficacy of the fatty acid, but did not affect the efficacy of the monoglyceride. Temperature had no effect on the antimicrobial efficacy of the compounds, but it was observed to be dependent on the nature of the strains.

3.2 DISCUSSION

Sugar esters have shown increased interest due to advantages with regard to environmental compatibility compared to petrol-derived standard products, as they are biodegradable and formed from renewable sources such as fatty acids and sugars (Ferrer *et al.*, 2005). With respect to their use as emulsifiers, an advantage of carbohydrates as the water solubilising hydrophilic component is their ability to offer a full range of HLB (hydrophilic lipophilic balance) values. This can be achieved by varying the carbohydrate itself (monosaccharide vs disaccharide, glucose vs galactose etc), and by controlling the degree of esterification. However, it is the antimicrobial potential of carbohydrate fatty acid derivatives, which has received less attention than their functional properties as emulsifiers or non-ionic surfactants, which is of interest in this study. There is limited information on the antimicrobial properties of CFA derivatives for use as food preservatives. This is in contrast to the extensive literature for monoglycerides, such as monolaurin, which is one of the most active antimicrobial fatty acid derivatives investigated to date (Conley and Kabara 1973; Bell and De Lacy 1987; Schlievert *et al.*, 1992; Ababouch *et al.*, 1994a; Blaszyk and Holley 1998; Chaibi *et al.*, 1998; Bergsson *et al.*, 2001; Ruzicka *et al.*, 2003; Sun *et al.*, 2003; Devulapalle *et al.*, 2004; Peterson and Schlievert 2006).

Previous studies on antimicrobial properties of sugar esters primarily focused on sucrose or other disaccharide ester derivatives (Hathcox and Beuchat, 1996). Some workers have pointed out that many of the studies were not carried out using regiochemically pure compounds (Devulapalle *et al.*, 2004), thus making it difficult to definitively ascribe antimicrobial activity to a specific chemical structure. Moreover, many studies were semi-quantitative at best and did not include reference or control

compounds that would allow comparison of efficacy with commercially available antimicrobial fatty acid derivatives. Thus, it was often difficult to ascertain from the literature whether novel antimicrobial fatty acid esters were better than existing commercial products for a given application. It was also difficult to compare the efficacy of these derivatives as synthesized by different groups.

The correlation of chemical structure with efficacy and/or mechanism of action has been difficult. Rios and Recio (2005) pointed out that many articles on natural products claim so-called "exciting" antimicrobial activities, despite major flaws in methodologies used. Most frequent amongst them are the lack of sound criteria for activity, the omission of appropriate in-test controls, the inclusion of unrealistically high assay dosages and the nature of the bioassay itself (selection of target organism, endpoints, *etc*).

There are several different assays for screening antimicrobial activity. Many publications report the antimicrobial activity of plants using general *in vitro* screening assays for microbial growth inhibition. The standard general screening assays include diffusion assays, dilution assays and bioautographic assays. Paper disc diffusion assays are generally used for screening of antibacterial and antifungal activities from natural extracts and compounds. However, the diffusion method is not appropriate for testing nonpolar samples or samples that do not easily diffuse into agar where a zone of inhibition diameter has to be measured (Cos *et al.*, 2006). For that reason, in the present study, a broth microdilution method was chosen.

Frequently, natural products are tested only against a single species or class of microorganisms. When the product exhibits high antimicrobial activity, it is then identified as a potential 'hit'. However, this 'narrow focus' strategy overlooks the

incidence of false-positives, which can easily be solved by inclusion of other microbial screens of bacteria, fungi, parasites and/or viruses (Cos *et al.*, 2006). Hence, in the present study a range of Gram positive and Gram negative bacteria from food or clinical isolates were included, focusing on *Listeria monocytogenes* and *Staphylococcus aureus*.

This study was primarily concerned with the synthesis of carbohydrate fatty acid derivatives for evaluation as antibacterial agents, with a view to discerning the impact of the following structural variations on antimicrobial efficacy:

(i) variation of the length of fatty acid chain (lauric and caprylic),

(ii) the type of fatty acid carbohydrate linkage (ester and ether),

(iii) the monosaccharide core (glucopyranoside, mannopyranoside or galactopyranoside),

(iv) the anomeric configuration of the sugar moiety (α or β glucopyranoside),

(v) the role of carbohydrate versus non-carbohydrate hydrophilic cores (carbohydrate and pentaerythritol laurates)

(vi) the degree of substitution (monoester or diester) of the carbohydrate.

In general, the CFA derivatives synthesized in this study were shown to be more effective against Gram positive than Gram negative bacteria ($p<0.05$). This trend was also observed for the fatty acid and monoglyceride controls, in accordance with previous studies (Conley and Kabara 1973; Bergsson *et al.*, 2002; Ruzicka *et al.*, 2003). We obtained similar MIC values of 10 μg/mL (0.04 mM) for monolaurin against *L. monocytogenes* as those reported by Wang and Johnson (1992), and Oh and Marshall (1993). We reported MIC values for lauric acid between 125 μg/mL (0.63 mM) and 250 μg/mL (1.25 mM), lower than those reported by Kitahara *et al.* (2004), of 400 μg/mL

against methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA, probably due to strain differences.

The MIC values (0.04 mM) of lauric derivatives **9a** (alpha lauric ether) and **4c** (lauric ester of mannopyranoside) against *Listeria* strains were found to be equivalent to that of monolaurin and in excess of that reported by Monk *et al.,* (1996), for a lauroyl-sucrose ester.

The importance of the chain length of the fatty acid ester was investigated using both lauric and caprylic derivatives. With respect to the effect of chain length on antimicrobial activity of the CFA derivatives, there was a difference in efficacy between Gram positive and Gram negative bacteria. Lauric acid and derivatives had higher activity against Gram positive bacteria, whereas caprylic acid and its ester derivative (compound **6a**) were more active than lauric acid derivatives against *E. coli* ATCC 25922 and *P. fluorescens.* This trend was also observed for the monoglyceride controls and is in accordance with general trends observed for medium and short chain fatty acids (Conley and Kabara, 1973).

The data obtained in this study is also comparable to that of Nair *et al.* (2004a), where populations of *L. monocytogenes* and *E. coli* O157:H7 were shown to decline below detection levels using 50 mM of MC or CA in bovine milk. The same authors, Nair *et al.* (2005b), described antimicrobial activity for both CA and MC and found that *Streptococcus* spp. were the most sensitive, and *E. coli* the most tolerant. Whilst both lauric and caprylic fatty acid derivatives retained good activity against Gram positive bacteria, only the caprylic acid derivative displayed useful efficacy against Gram negative bacteria.

The enhanced efficacy of the shorter chain fatty acid over the medium chain fatty acid could be attributed to differences in the outer membrane structure and permeability between Gram negative and Gram positive bacteria. The outer membrane of Gram-negative bacteria behaves as a entry barrier against fatty acids, but the cell wall of Gram-positive bacteria may adsorb and transport fatty acids into the inner membrane (Galbraith and Miller, 1973b; Kitahara *et al.*, 2004).

A detailed study by Ruzin and Novick (2000) showed the rapid hydrolysis of ML with liberation of lauric acid ($t_{1/2}$ of ~5 min) in *S. aureus* cultures. The Geh lipase (glycerol ester hydrolase) was found to be responsible for approximately 80% of the monolaurin hydrolysing activity in the supernatant, but the same authors also reported cell-associated lipolytic activity. This activity might be explained by the action of either intracellular or membrane-bound lipases. However, intracellular lipases, in *S. aureus*, are not expected to play any significant role in ML degradation, since, ML is a surfactant and is considered unlikely to reach the cytoplasm, therefore they suggested that *S. aureus* had one or more membrane bound esterases that participated in the hydrolysis of monolaurin and release of lauric acid. 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 by extracellular or membrane-bound enzymes or whether their mode of action might depend on such hydrolysis. The clarification of the role of the free fatty acid in antimicrobial efficacy was related to the design of compounds with an ester or an ether glycoconjugate linkage.

Reporting on the antimicrobial efficacy of ether and ester glyceride compounds, Isaacs *et al.*, (1995), suggested that ether lipids should remain antimicrobial for a longer period of time than monoglycerides with ester linkages, due to their greater stability in biological systems. The current work suggests that while membrane bound or free esterases may cleave ester bonds of a glycerol or a carbohydrate fatty acid derivative, the ether carbohydrate fatty acid derivatives retained higher activity than the ester derivatives, as the ether linkage is not readily hydrolysed by esterases in biological systems (Ved *et al.*, 1984), and that the release of a free fatty acid may not be required for antimicrobial activity. This is in agreement with Isaacs and Lamp (2000), who 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.

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.

In an effort to probe the role of the carbohydrate moiety in antimicrobial efficacy, ester and ether fatty acid derivatives of the following carbohydrates were synthesized and tested: α-glucose, β-glucose, α-mannose and α-galactose. Of these, differences in efficacy were measured for compounds with the same glycoconjugate bond and alkyl chain length (compounds **4a, 4b, 4c** and **4d**). The results showed that the lauric ester derivative of methyl β-D-glucopyranoside (**4b**) and methyl α-D-mannopyranoside (**4c**), showed higher activity than any other ester derivatives against *Listeria* spp. and *S.*

aureus strains, indicating that the nature of the carbohydrate moiety plays a role in the antimicrobial efficacy of CFA derivatives.

From these studies we conclude that the sugar itself may be a factor in determining efficacy. This is consistent with the findings of Watanabe *et al.*, (2000) who also concluded that the configuration of the hydroxyl group in a carbohydrate moiety markedly affected antibacterial activity. In these studies, we also found that a minor structural change in the carbohydrate may have a major influence on the solubility of a compound in water. For example, compounds **4a**, **4b**, and **4c** (α-glucose, β-glucose, α-mannose) were soluble, whereas the structurally similar compound **4d** (α-galactose) was practically insoluble. This further highlights the importance of the choice of carbohydrate moiety for antimicrobial efficacy of CFAs.

The differences observed in this study between compounds with ester or ether bonds of the same carbohydrate fatty acid (compounds **4a** and **9a**; and compounds **4b** and **9b**) show that the nature of the bond between the fatty acid and the sugar also has an influence on antimicrobial activity. Compound **9b** (β lauric ether) was less inhibitory than the free fatty acid (LA) and monoglyceride (ML) against *Listeria* spp. and *S. aureus* strains. In some cases, compound **9a** (α lauric ether) had an enhanced activity by comparison with compound **4a** (α lauric ester) and **4b** (β lauric ester), particularly for *Listeria* spp. However, the same compounds had comparable levels of efficacy against Gram negative bacteria.

Further evidence of a role for the carbohydrate moiety in CFAs efficacy came from data for the lauric ester anomers of methyl glucopyranoside **4a** and **4b**. When these compounds were tested against *S. aureus*, the beta configuration showed higher activity, whereas against *Listeria* strains similar activity for both derivatives was observed. The

lauric ether anomers of methyl glucopyranoside **9a** and **9b** also showed a marked difference in activity when tested against *S. aureus* and *Listeria* spp., with the alpha configuration showing higher activity for both strains (MICs 0.04 mM to 0.08 mM) compared to the beta configuration (MICs 1.25 mM to 10 mM), $p<0.05$.

In general, the alpha configuration of the carbohydrate moiety of the compounds synthesized was more effective than the beta, for both ester and ether derivatives of the same carbohydrate. This further indicates that the carbohydrate can have a role in the antimicrobial efficacy of the carbohydrate fatty acid derivative.

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 **9a** (α-glucose lauric ether), was far more active against most *S. aureus* and *Listeria* strains than compound **9b** (β-glucose lauric ether). The reasons for this discrepancy were not clear from these studies and required further investigation of the mechanism of action of these compounds.

The non-carbohydrate pentaerythritol monoester **14**, which has the same number of free hydroxyl groups as the carbohydrate monoester derivatives (**4a-d**), showed negligible activity against both microorganisms tested (*S. aureus* ATCC 25923 and *E. coli* ATCC 25922), indicating that the carbohydrate structure might play an important role in the antimicrobial activity of these compounds.

We found that not only were free single or multiple hydrophilic groups necessary for biological activity, as observed by Conley and Kabara (1973), but that the overall structure of the hydrophilic moiety conjugated to the fatty acid is important for antibacterial activity. For example, the antimicrobial activity associated with the lauroyl

pentaerythritol monoester **14** with three free hydroxyl groups was negligible compared to compounds **4a**, **4b** and **4c** which also had the same number of free hydroxyl groups. This could be related to the more highly constrained nature of the ring structure of the carbohydrates and the associated entropic benefit upon binding to a biological receptor, compared to the more flexible pentaerythritol structure. It could also be related to the fixed orientation of the hydroxyl groups of the carbohydrate, and the increased likelihood of recognition of a carbohydrate derivative by a biological receptor compared to a pentaerythritol derivative. However, this remains speculative until further evidence for the mechanism of action of these compounds is realised.

The degree of substitution of these derivatives was also shown to be crucial as both the non-sugar pentaerythritol diester **15** and the carbohydrate methyl α-D-glucopyranoside diester **12a** were much less soluble in water than the corresponding monoesters. As a consequence, no antimicrobial activity data for these compounds could be obtained. Results for diester **12a** demonstrate that more than one fatty acid esterified to a monosaccharide render the compound insufficiently soluble for antimicrobial activity evaluation by the methodology chosen for this study. While this is a limiting factor for more general applications of these compounds, such low solubility as found for compounds **4d, 12a** and **15** would limit their potential for application in food systems as antimicrobials in any case.

Combinations of sub-MIC preservatives with other minimal 'hurdles' may contribute to the control of microbiological issues in food systems while minimizing sensory and quality impacts on a food (Gutierrez *et al.*, 2008). Thus, in this study we evaluated the extent to which sub-MIC concentrations influenced bacterial growth.

The half maximum inhibitory concentration (IC_{50}) was defined as the concentration required for 50% inhibition of growth. For some compounds, like monolaurin and the lauric esters, the inhibition of growth was very effective at almost all concentrations tested, making it difficult to calculate an IC_{50} due to the insufficiency of data points. For that reason, future studies should encompass a wider range of low concentrations for both control and novel compounds.

IC_{50} values obtained, showed similar trends for lauric and caprylic acid efficacy to those recorded for MICs. Although, differences in the degree of sensitivity between strains were observed for *L. innocua* NCTC 11288 and *L. monocytogenes* ATCC 7644; and for *S. aureus* ATCC 25923 and *MRSA* ATCC 33591. This observation reinforces the importance of including a range of bacteria species to avoid the incidence of false-positives as potential 'hits'.

The data obtained from the increase in lag phase and decrease in maximum specific growth studies showed that sub-MIC concentrations can also modify bacterial growth significantly. Antimicrobial efficacy of compound **9b** (beta lauric ether) was considerably lower than that of the other derivatives, but these results show that nevertheless, there is a significant effect of this compound on bacterial growth, even at sub-MIC concentrations. Nair *et al.*, (2004b) also observed this behaviour using monocaprylin (50 mM) which reduced *Enterobacter sakazakii* growth in reconstituted infant formula by >5 log CFU/ml at 37°C, whereas approximately 1.5 log CFU/ml of the pathogen survived after 24 h of incubation using half the concentration of antimicrobial.

The reduction in population density and the increase in the lag phase observed when strains were incubated at different pH levels, indicates that pH had an effect on bacterial growth and on antimicrobial efficacy of the fatty acid, but did not affect the

efficacy of the monoglyceride. No growth was observed at any concentration of lauric acid tested at pH 4, whereas, growth was observed at the ½ MIC of monolaurin at all pH levels tested after 48 hours. This is in accordance with the study reported by Sun *et al.*, (2003), who observed that bactericidal potency of monoglycerides, unlike that of free fatty acids, does not show any variation over a broad pH range, as these molecules remain un-ionised. However, Oh and Marshall (1993a) demonstrated that at 35°C, *L. monocytogenes* grew well in the presence of 18 μM monolaurin in a medium adjusted (with HCl) to pH 5.5. However, when the pH was further reduced to pH 5.0, the bacterium did not grow.

The enhanced efficacy of lauric acid against *S. aureus* ATCC 25923 at pH 5 could be explained as follows; at low pH bacterial cells take up fatty acids and monoglycerides at a higher rate which reduces the bacterial membrane-medium interfacial tension, thereby resulting in an enhanced inhibitory effect on the target bacterium (Galbraith and Miller, 1973a; Oh and Marshall. 1992).

In this study, temperature seemed to have no effect on the antimicrobial efficacy of the fatty acid and its derivatives, but it was observed to be more dependent on the nature of the microorganisms, as the sensitivity of the bacteria could differ depending on the storage temperature. The antimicrobial effect was more marked at higher temperatures (35°C and 20°C) than at lower temperatures (10°C and 4°C). This was expected because the microorganisms were mesophilic in nature, whereas at refrigeration temperatures, the metabolism of the microorganisms becomes slower; therefore, growth and death rates are lower. Nair *et al.* (2005a), observed that monocaprylin had a markedly increased antibacterial effect at room temperature, which again could be attributed to the mesophilic nature of *E. coli* O157: H7.

The significantly reduced antibacterial effect of fatty acid and derivatives at 4°C can also be attributed to the decreased solubility of lipids at lower temperatures (Nair *et al.*, 2004b). The reduced antibacterial effect of fatty acid and monoglyceride at lower temperatures could also be due to the changes in the fatty acid profile and fluidity of the bacterial cell membrane at refrigeration temperatures. Electron microscopic examination of bacterial cells exposed to monoglycerides has revealed plasma membrane disintegration, leaving the peptidoglycan cell wall intact (Bergsson *et al.*, 1998). Changes in cell membrane lipid composition, including alterations in the relative proportions of different fatty acid classes, and increased lipid unsaturation and fluidity upon exposure to refrigeration temperatures have been reported (McElhaney, 1976; Russel, 1984). These changes in the bacterial cell membrane may interfere with the action of fatty acid and monoglycerides, resulting in a reduced antibacterial effect.

The results obtained for *Pseudomonas* show that even at low temperatures lauric acid could be active against spoilage microorganisms, at MIC values, after 4 days. This is of interest for potential application studies with respect to control of spoilage of refrigerated fresh food products, many of which can be associated with *Pseudomonas* spp.

4. MECHANISM OF ACTION STUDIES

Following the antimicrobial activity studies, in which the CFA derivatives synthesised demonstrated interesting antimicrobial activity against certain bacteria, and where the efficacy was to a certain extent related to compound structural differences, this study seeks to further our knowledge through extending the investigation of the structure/activity relationships and mode of action of CFA derivatives.

It has been proposed that the cell membrane is the principal site of action of fatty acids and their esters. A detailed study by Ruzin and Novick (2000) showed that monolaurin inhibited the production of exoenzymes and virulence factors in *S. aureus* at sub-MIC concentrations. Moreover, they were able to show rapid hydrolysis of monolaurin with liberation of lauric acid ($t1/2$ of ~5 min) in the presence of *S. aureus* cells, suggesting that the prolonged inhibitory effects associated with monolaurin might be due to liberation of lauric acid by hydrolases present in the cell preparation. However, they were not able to rule out the possibility that monolaurin itself might have some inhibitory action prior to its hydrolysis.

These findings raised important questions for CFA derivatives. 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. Results in chapter 3 showed that an ether analog of a CFA (compound **9a**) retained higher or similar antimicrobial activity to the ester derivative, thus indicating that the release of a free fatty acid was not an absolute requirement for antimicrobial activity since ethers will not be hydrolysed by enzymes present in cell preparations.

4.1 RESULTS

4.1.1 Degradation of CFA derivatives monitored by thin layer chromatography (TLC)

Following a report by Ruzin and Novik (2000), which described a TLC method to determine the fate of monolaurin in bacterial cultures, a modified method was developed using *S. aureus* ATCC 25923 grown in the presence of the CFA derivatives.

Section 2.6 described in detail the development of this method. Optimum results were obtained using pure ethyl acetate as the mobile phase and a vanillin solution as the staining agent. Figure 4.1 shows a chromatogram of pure compounds analysed by this TLC method.

Figure 4.1. TLC plate developed with pure ethyl acetate and stained with a vanillin solution. 1. Lauric acid (LA); 2. α ester: Methyl 6-O-lauroyl-α-D-glucopyranoside (compound **4a**); 3. α ether: Methyl 6-O-dodecanyl-α-D-glucopyranoside (compound **9a**) and 4. gluc: Methyl-α-D-glucopyranoside (**1a**).

It appeared that the TLC was suitable as a method to distinguish free fatty acids from their ester and ether derivatives. However, when attempts were made to use this assay to monitor the degradation of CFAs in cell suspensions it was not successful. This was possibly due to the low concentration of compounds in the broth. It was concluded that the method was not sufficiently sensitive for use.

Due to time limitations, these experiments were not pursued further. An alternative approach, using more advanced analytical techniques is required to monitor the degradation of CFAs in cell suspensions. Techniques such as LC-NMR (Liquid Chromatography - Nuclear Magnetic Resonance) and LC-MS (Liquid Chromatography - Mass Spectrometry) may be more useful. Following appropriate sample preparation, these techniques could separate the desired analytes by HPLC (High-Performance Liquid Chromatography), and when coupled with a Mass Spectrometer or a Nuclear Magnetic Spectrometer, could unambiguously identify the analyte. In addition, these techniques have limits of detection considerably lower than the TLC method employed.

4.1.2 Mode of action studies

4.1.2.1 Leakage of material absorbing at 260nm

Nucleic acid and its related compounds, such as pyrimidines and purines, are known to absorb UV light at a wavelength of 260 nm. The presence of these materials in a suspension can be used as an indicator of damage to the cell membrane.

Leakage was determined using nisin as a control compound known to cause membrane damage. Table 4.1 shows the changes in OD_{260} readings for all strains and compounds tested for up to 120 min of exposure. The five bacterial strains showed similar patterns in their release of material absorbing at 260 nm. When *S. aureus*

suspensions were treated with Nisin, LA, ML and CFA derivatives using concentrations of four times their respective MICs it was observed that the amount of UV absorbing substances released increased as the time of exposure increased. OD_{260} increased rapidly at first, becoming more gradual after 60 minutes (Figure 4.2). An exception was observed for compound **9b**, the β-glucose lauric ether, which even after 120 min of exposure did not show any significant increase in material absorbing at 260 nm (Figure 4.2), indicating a lack of effect on the cell membrane.

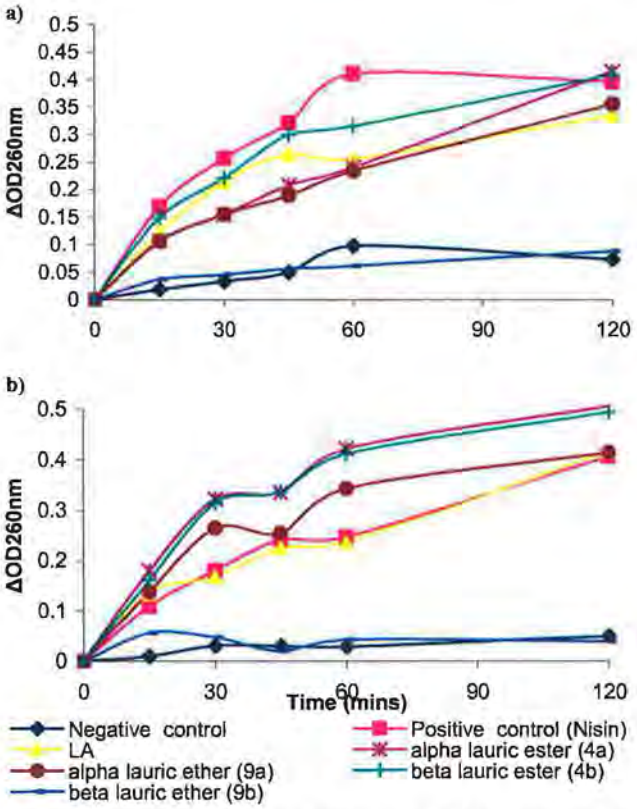


Figure 4.2 Effect of test compounds on release of UV absorbing substances from: a) *S. aureus* ATCC25923, b) *S. aureus* ATCC43300 (MRSA).

These results were not conclusive in establishing a unique or distinctive mode of action for the CFA derivatives with structural differences. All of the derivatives gave

broadly similar profiles for release of OD_{260} absorbing substances with the exception of the beta ether derivative which was similar to the negative control. The beta ether (compound **9b**) data was consistent with its antimicrobial efficacy. Thus, we can conclude that membrane damage is clearly an aspect of the mode of action of CFAs. Consequently further and more specific mode of action studies were considered. Moreover, it is advisable to use multiple methods to determine diverse mechanisms of action of these derivatives and to establish trends.

Table 4.1 Effects of CFAs on membrane integrity of *S. aureus* strains measured by release of UV absorbing components at 260 nm.

Organism	Time (mins)	Negative control	Positive control (Nisin)	Fatty acid (LA)	Monoglyceride (ML)	Carbohydrate fatty acid derivatives			
						4a	9a	4b	9b
S. aureus	0	0	0	0	0	0	0	0	0
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

4a. Methyl 6-O-lauroyl-α-D-glucopyranoside; 9a. Methyl 6-O-dodecanyl-α-D-glucopyranoside; 4b. Methyl 6-O-lauroyl-β-D-glucopyranoside; 9b. Methyl 6-O-dodecanyl-β-D-glucopyranoside. ± figures next to each result indicate standard deviation values.

4.1.2.2 Modified Live/Dead BacLight assay

Bacterial membrane damage was further assessed using the Live/Dead® *Bac*Light™ viability assay. The fluorescence intensities of the stained bacterial suspension at 535nm (G, green) and 615nm (R, red) represent live and dead cells, respectively. A standard curve was constructed by plotting the fluorescence G/R ratios obtained from preparations of known live:dead cell ratios, which were calculated by dividing the green and red intensities (Figure 4.3). The results from control staphylococcal cells and cells treated with the CFA derivatives or standards are shown in Figure 4.4 and are expressed as the percentage of results obtained with control cultures not exposed to the antimicrobial compounds using the standard curve.

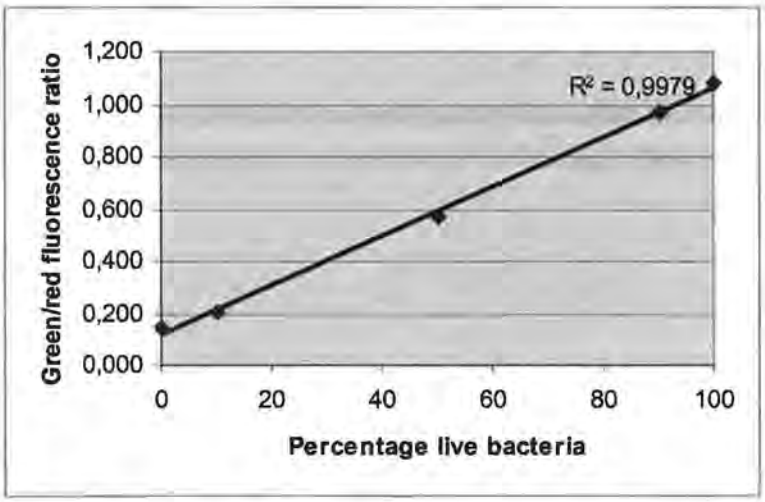


Figure 4.3. Standard curve for the live and dead cells fluorescence G/R ratio

Exposure of staphylococcal cells to CFA derivatives showed altered cytoplasmic membrane permeability for lauric acid and the alpha compounds. Both alpha lauric ester (compound **4a**) and the alpha lauric ether (compound **9a**), had a low fluorescence ratio,

corresponding to 10-20% viability by comparison with the untreated control (Figure 4.4).

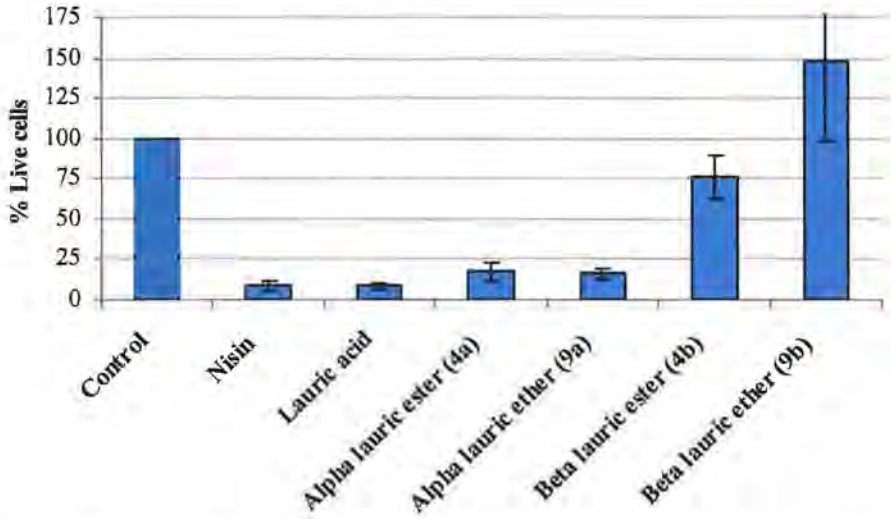


Figure 4.4. Effect of CFA derivatives on *S. aureus* ATCC 25923 membrane integrity measured using the BacLight assay. Error bars represent standard deviation.

On the other hand, the beta lauric ester (compound **4b**) derivative showed a higher fluorescence ratio, corresponding to retention of approximately 75% viability. The beta ether derivative (compound **9b**) did not significantly affect the fluorescence ratio, suggesting that this compound did not cause membrane damage, which was in agreement with the observation for the leakage studies at 260nm for this compound.

4.1.2.3 BacTiter Glo assay

The BacTiter-Glo™ Microbial Cell Viability assay was used to determine the number of viable bacterial cells in a culture based on quantitation of the ATP present. 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 4.5. Monolaurin and the alpha lauric derivatives (compounds **4a** and **9a**) resulted in a significant decrease in ATP levels, of around 99%, and were comparable to the results achieved with nisin as the positive control for membrane disruption. For lauric acid and both beta derivatives (compounds **4b** and **9b**), there was also a significant decline in ATP detected, with a decrease around 70-80%, but to a lesser extent than that observed with the alpha lauric derivatives. Again, this reinforces the importance of the anomeric configuration of the sugar moiety in antimicrobial efficacy.

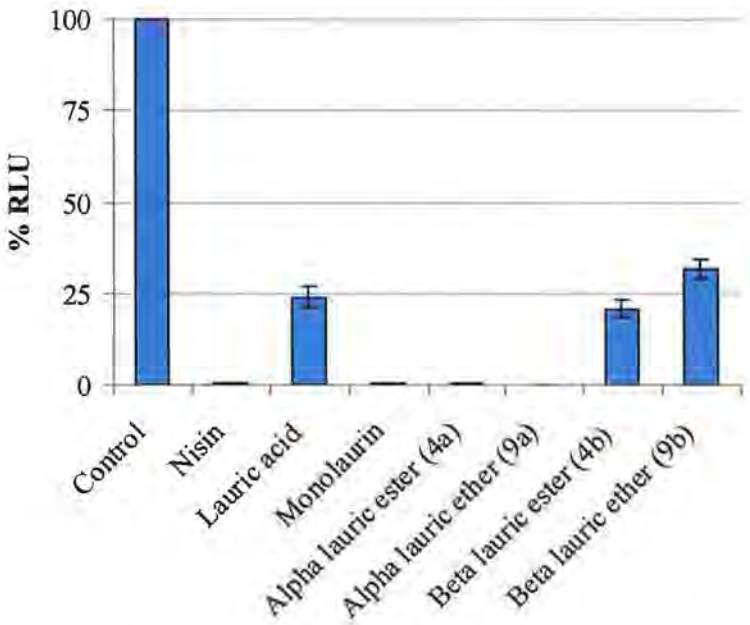


Figure 4.5. Antimicrobial activity of CFA derivatives using the BacTiter-Glo™ assay. Values are expressed as a percentage of those obtained with control cultures not exposed to the antimicrobial compounds. Error bars represent standard deviation.

4.1.2.4 Phenotype MicroArray

Phenotype MicroArray (PM) analyses were used to assess differences in bacterial metabolic activity and phenotypic response between untreated control cells and cells treated with selected CFA derivatives (PM 1-10); as well as their interactions with known antimicrobials (PM inhibitors, PM 11-20). This allowed us to further differentiate structure-related responses for different CFAs. This technology couples electron transport to the reduction of a tetrazolium dye and thereby provides a direct colourimetric measurement of respiration or growth on a variety of substrates. Note that respiration may be a direct reflection of growth.

Data presented here were based on utilization *vs.* non-utilization of PM sources by *L. monocytogenes* ATCC 7644 cells with or without CFA derivatives. Kinetic curves for each test well were constructed to evaluate the response of the control cells to each PM source and compared with the response of cells treated with CFA derivatives. The full range of kinetic curves constructed can be found in Appendix 2.3 and 2.4.

The data analysis focussed on key activity trends between compounds, possibly related to the glycoconjugate linkage or anomeric configuration, and possible antimicrobial mechanisms of action.

The following figures represent significant results from PM plates where a notable difference between control and/or treated cells was observed. The Omnilog units represent values based on the height of the curve and may be interpreted as cell growth. An example featuring the utilization of particular carbon sources (positive response) is shown in Figure 4.6.

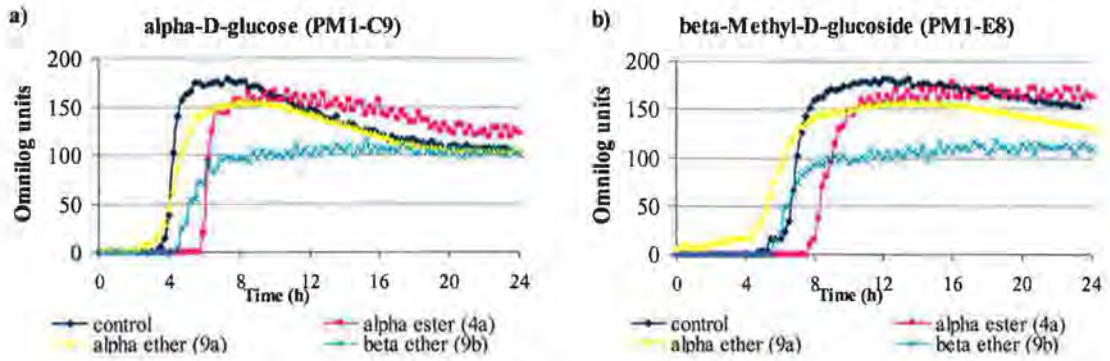


Figure 4.6 Utilization of PM carbon sources by *L. monocytogenes* ATCC 7644.

The above figure (Figure 4.6) shows a positive response of *L. monocytogenes* ATCC 7644 for the two PM carbon sources, a) α-D-glucose and b) β-Methyl-D-glucoside, which were also constituents of the CFA derivatives synthesised. No growth promoting effect was observed in the presence of the CFA derivatives. Moreover, the alpha ester derivative (compound **4a**) increased the lag phase associated with PM source utilisation and the beta ether derivative (compound **9b**) had a negative effect on the PM source utilisation which is similar to a reduction in growth.

In Figure 4.7.a it was observed that *Listeria* utilized the PM source thymidine, but to a lesser extent by comparison with α-D-glucose, PM1-C9 (Figure 4.6.a). The addition of the ether derivatives (compounds **9a** and **9b**) did not significantly change the growth (PM source utilisation) compared to the control. However, the addition of the ester derivative (compound **4a**), increased the lag time up to 24 hours. A similar trend was observed for D-tagatose and 2,3-butanone (Figure 4.8.e-f), but here, additionally, the alpha ether (compound **9a**) enhanced the growth of the microorganism and a difference between the ether derivatives was observed, suggesting an anomeric configuration effect.

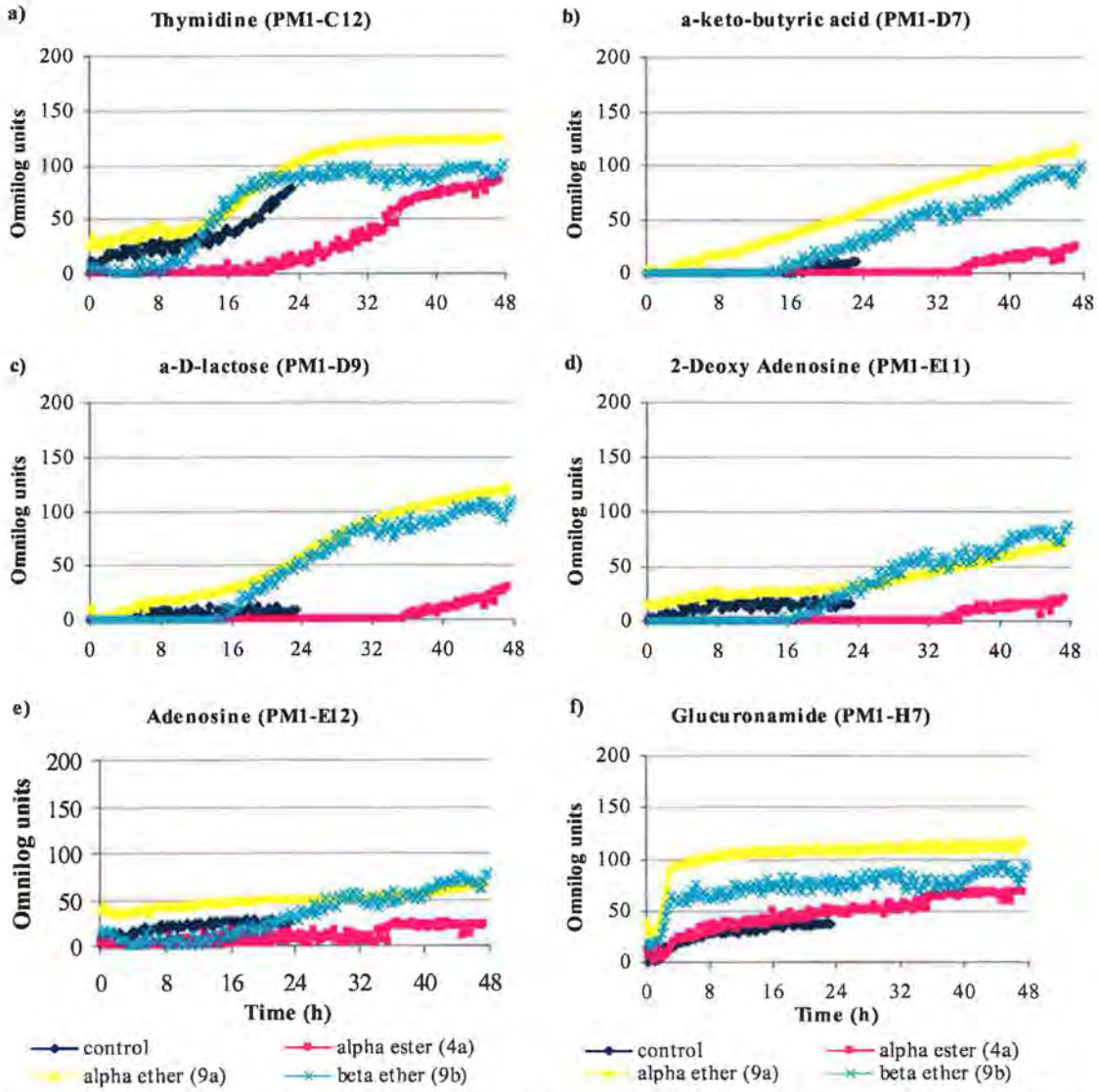


Figure 4.7 *L. monocytogenes* ATCC 7644 cells with and without CFA derivatives grown in PM1 Carbon sources

Figure 4.7.b-d show that the microorganism did not use these PM sources particularly well up to 24 hours. For comparison where PM sources were well utilised refer to PM1-C9 (Figure 4.6.a), which is evidenced by the utilisation growth curve for this well. The addition of the alpha ester (compound **4a**), did not indicate any significant change by comparison with control. However, with the addition of the ether derivatives

(compounds **9a** and **9b**), the utilisation of these PM sources increased, primarily after 24 hours, by comparison with the inhibition of the utilisation of this PM source in the presence of the alpha ester (compound **4a**). Thus, the trend of alpha ester having greater antimicrobial efficacy against this microorganism was retained. A similar trend was observed for adenosine as a PM source (Figure 4.7.e), but in this case, only the beta ether (compound **9b**) seemed to promote the utilisation of the PM source after 24 hours.

A different trend was observed for glucuronamide (Figure 4.7.f), where the microorganisms' response was greater from the beginning in the presence of this PM source, and was also enhanced in the presence of both ether derivatives (compounds **9a** and **9b**). It may be that glucuronamide in the presence of the CFA derivative becomes more utilisable itself. The opposite case where in the presence of the PM source, the CFA derivative became metabolisable is unlikely. In the case of the ester compounds, it would be likely due to the release of the carbohydrate moiety through hydrolysis, however this would render the fatty acid free, which would lead to antimicrobial activity. No such hydrolysis is possible with the ether derivatives.

The carbon PM sources, β-D-allose, 3-0- β-D-galactopyranosyl-D-arabinose and 3-methyl-glucose, in Figure 4.8.a, c, d, had a similar trend to that observed for α-keto-butyric acid, α-D-lactose and 2-deoxy adenosine (Figure 4.7.b-d), where an effect of glycoconjugate linkage was noted. The alpha ester (compound **4a**) retained greater antimicrobial efficacy where the utilisation of the PM sources was inhibited; but at the same time, there was a difference for the response in the presence of the ethers for these PM sources, thus indicating that anomeric configuration is also important.

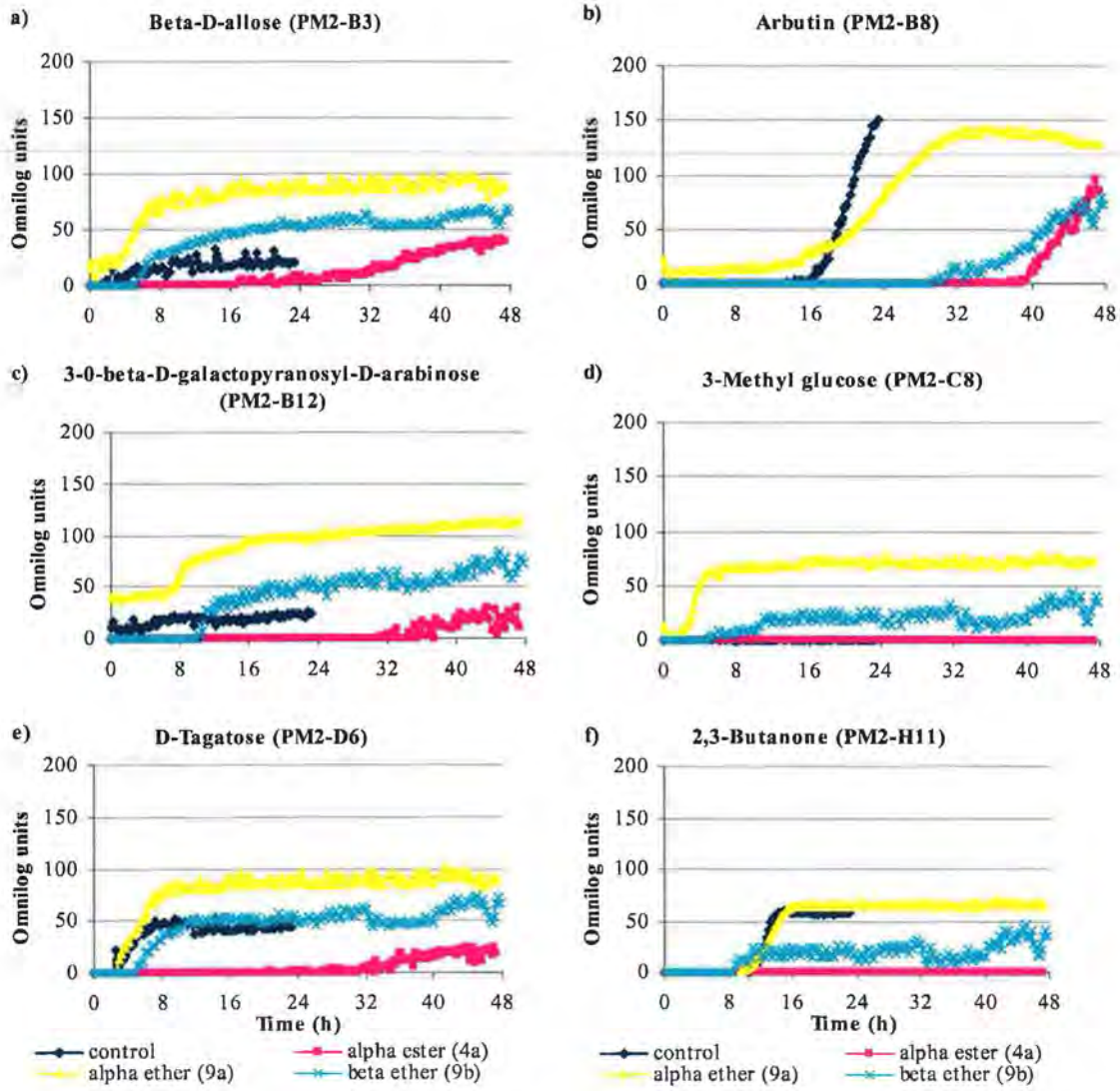


Figure 4.8 *L. monocytogenes* ATCC 7644 cells with and without CFA derivatives grown in PM2 Carbon sources

The utilisation of arbutin PM source (Figure 4.8.b) was minimal, even for control wells. After 16 hours it seemed that a shift in metabolism occurred and thereafter utilisation was strong. This trend was also evident in the presence of the alpha ether (compound **9a**). However, when the alpha ester (compound **4a**) and beta ether (compound **9b**) were present, the utilisation was inhibited for up to 32 hours.

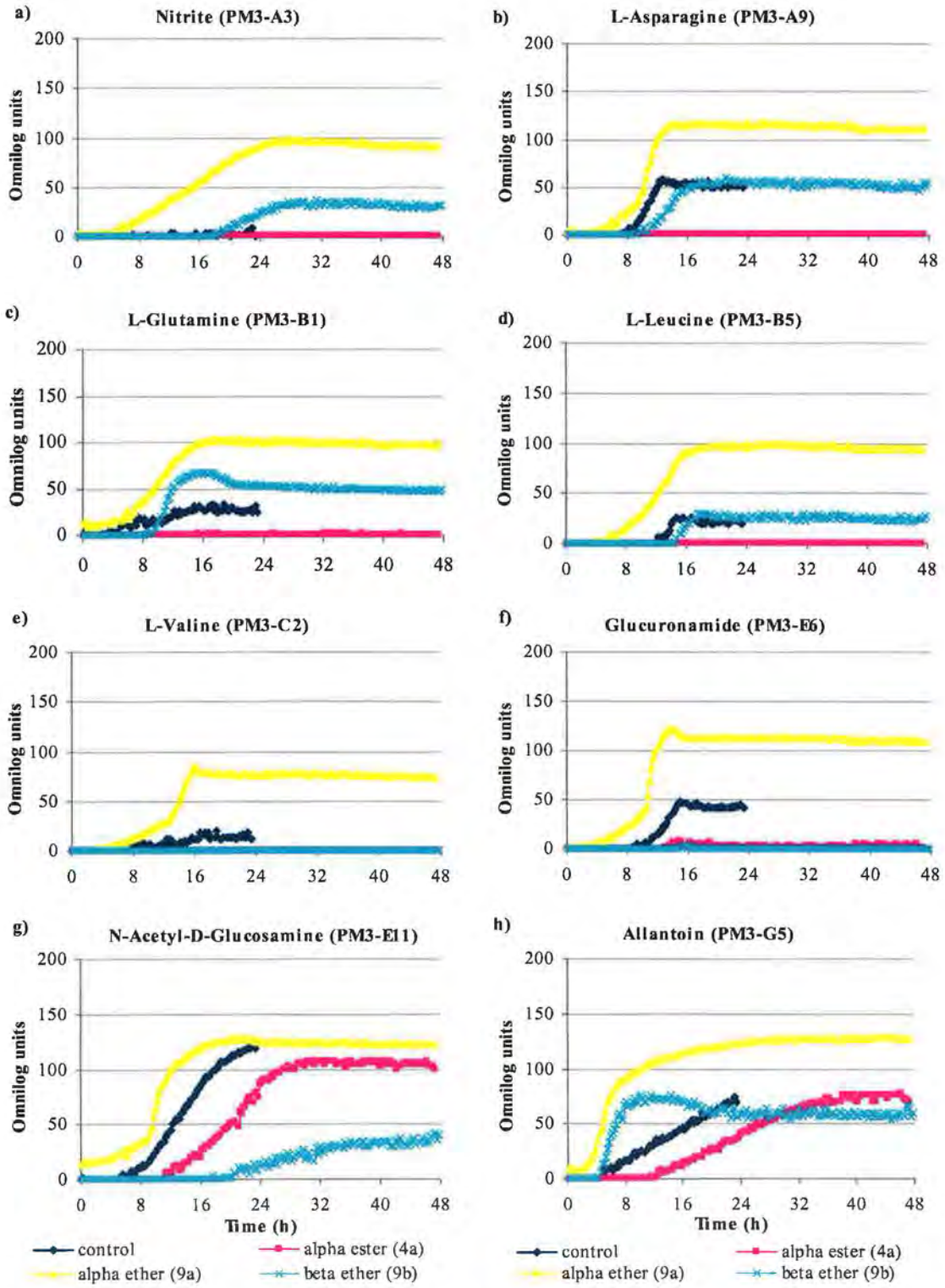


Figure 4.9 *L. monocytogenes* ATCC 7644 cells with and without CFA derivatives grown in PM3 Nitrogen sources

For nitrogen PM plate 3 (Figure 4.9.a-h), a general trend was obvious, where the alpha ether (compound **9a**) promoted the growth or utilisation of the PM sources. Again the alpha ester (compound **4a**) retained the greatest antimicrobial efficacy in the presence of nitrite, L-asparagine, L-glutamine, L-leucine, L-valine and glucuronamide as PM sources (Figure 4.9.(a-f). For complete nitrogen PM sources utilisation wells refer to Appendix 2.3, where the same trends were recorded for PM3-wells A2, A3, A7, A9, B1, B4, B5, B11, C2, C9, C11, E6, G7 and H6.

Furthermore, the beta ether (compound **9b**) was more inhibitory to PM source utilisation by comparison with control in the presence of L-valine, glucuronamide and N-acetyl-D-glucosamine (Figure 4.9.e-g).

However, in the antimicrobial efficacy studies (Chapter 3), the beta ether (compound **9b**) was not a promising candidate. This may perhaps support the hypothesis of an alternative site of action, as proposed in conjunction with the trends observed in lag phase and OD260nm studies. Additionally, this was one of the few instances where the alpha ester (compound **4a**) did not inhibit the utilisation of a PM source (N-acetyl-D-glucosamine and allantoin) by the *Listeria* (Figure 4.9.g-h).

For phosphorus and sulphur sources, PM plate 4 (Figure 4.10.a-h), similar trends were observed as noted for the nitrogen sources, in which alpha ether (compound **9a**) promoted growth or the use of the PM source and, the alpha ester (compound **4a**) again retained the greatest antimicrobial efficacy in the presence of these PM sources, phosphate, D,L-α-glycerol phosphate, phosphocreatine, tetratione and dithiophosphate (Figure 4.10.a, c, e, f, g). For complete nitrogen PM sources utilisation wells refer to Appendix 2.3, where the same trends were recorded for PM plate 4 in wells A4, B5, B6, D4, D5, E1, F3, F9, G1, G2, G8, H2 and H4.

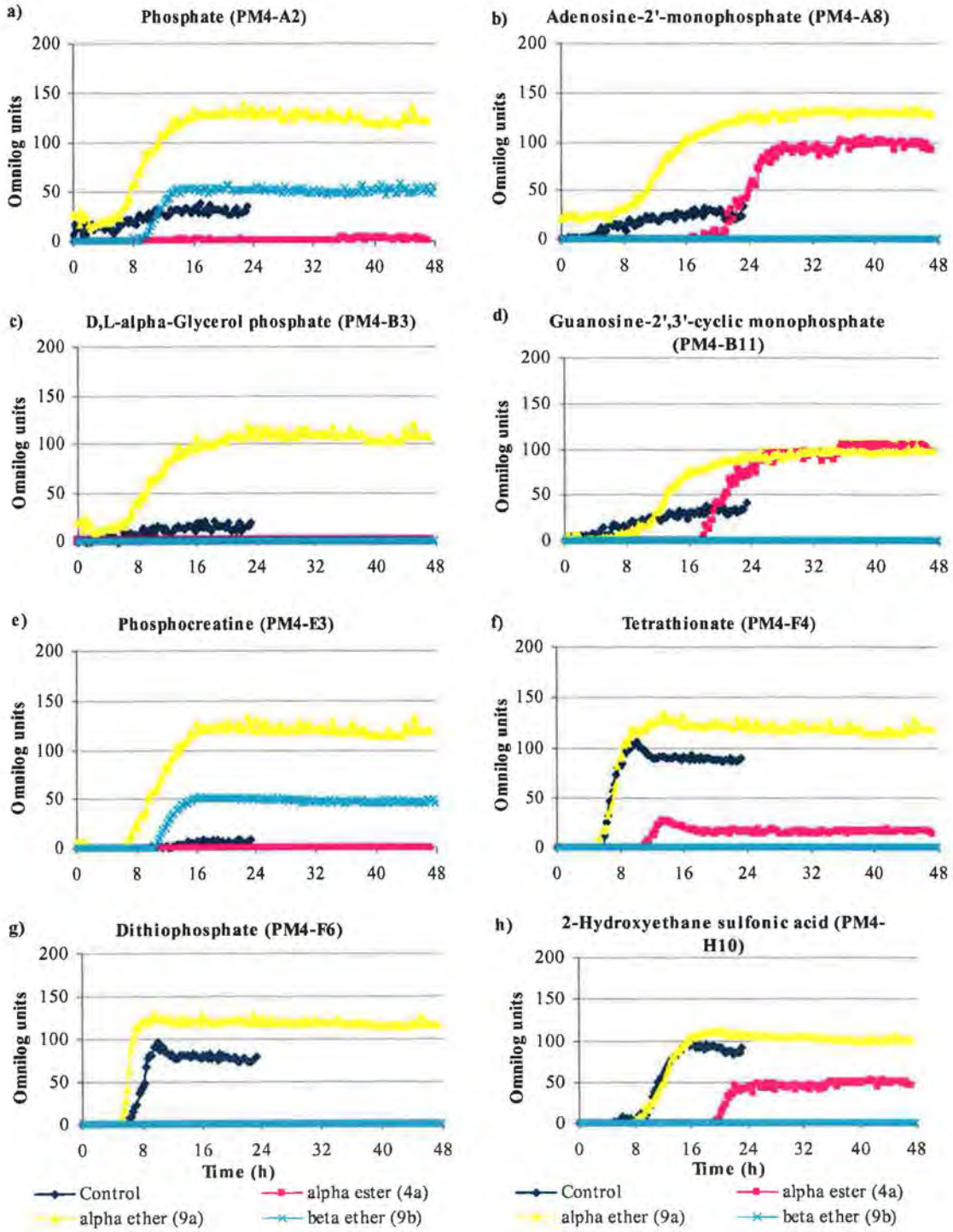


Figure 4.10 *L. monocytogenes* ATCC 7644 cells with and without CFA derivatives grown in PM4 Phosphorus and Sulphur sources

The presence of the beta ether (compound **9b**), which is normally a poor inhibitor of growth, here again prevented growth or utilisation of the PM sources by *Listeria*. Hence, confirming that anomeric configuration played a distinct role as alpha ether and beta ether derivatives show different trends for the same PM source.

It was also observed that growth in the control well, in the absence of CFA derivatives, was not particularly good up to 24 hours in the presence of nucleotides as PM sources, whereas in the presence of alpha ether (compound **9a**), the utilisation of the PM sources was enhanced, as previously seen for other PM wells. However, in this instance, the alpha ester (compound **4a**) was also associated with a strong utilisation of the PM source after 16 hours (Figure 4.10.b,d).

For peptide nitrogen sources, PM plate 6 (Figure 4.11.a-f), different trends within the plate were observed. For example, the growth in the control well containing Alanine-Asparagine (Figure 4.11.a) was strong up to 24 hours. In the presence of the ether derivatives, these PM sources were utilised but to a lesser extent, and as observed previously, in the presence of alpha ester (compound **4a**), an increased lag phase was recorded. Similar trends were observed for Alanine-Threonine and Arginine-Glutamic acid (Figure 4.11.b, c), where the alpha ester (compound **4a**) was completely inhibitory with no utilisation of the PM source evident up to 48 hours.

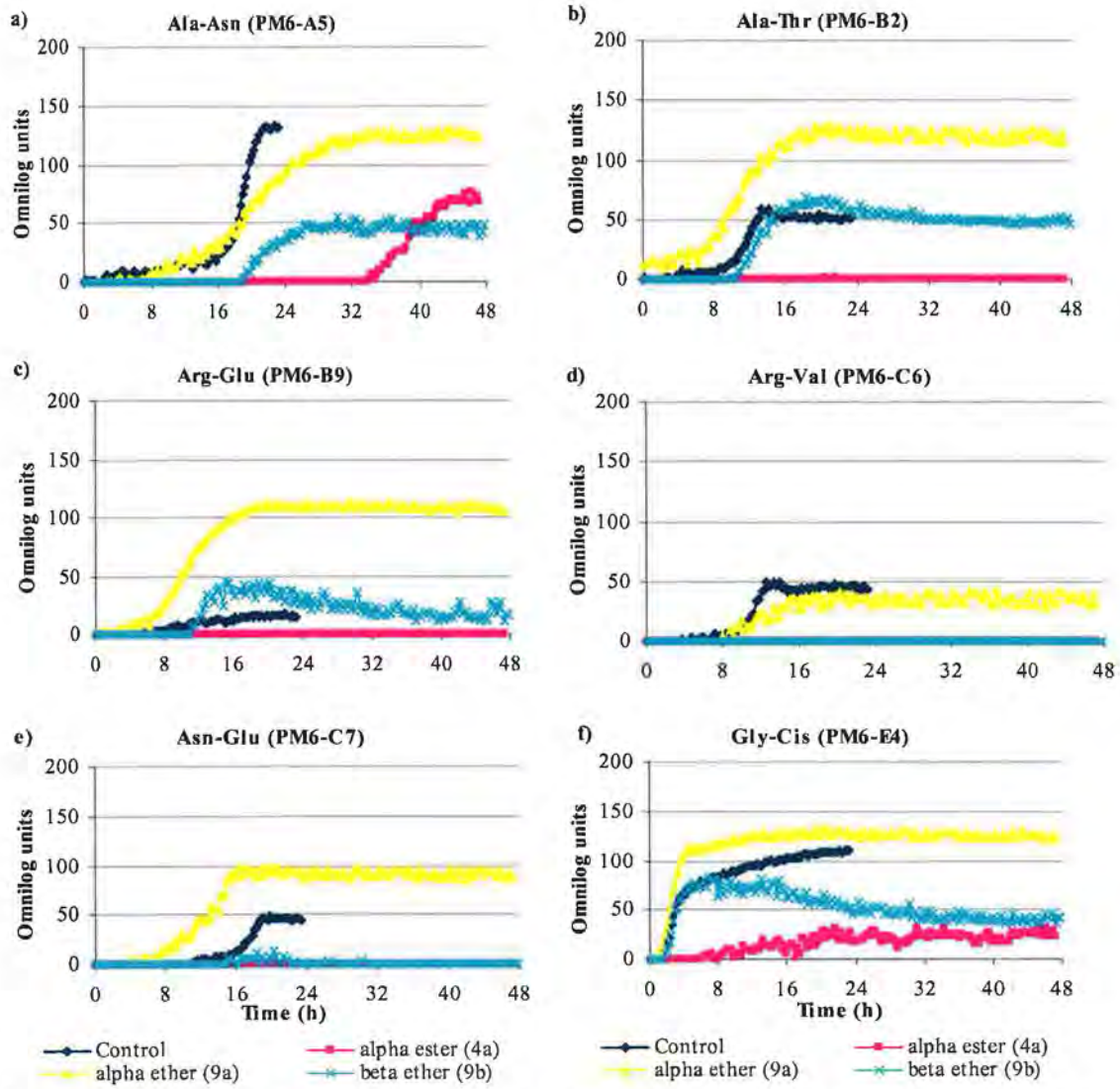


Figure 4.11 *L. monocytogenes* ATCC 7644 cells with and without CFA derivatives (4a, 9a, 9b) grown in PM 6 Peptide nitrogen sources

For other PM sources including Arginine-Valine and Asparagine-Glutamic acid, the alpha ester (compound **4a**) and beta ether (compound **9b**) inhibited utilisation strongly by comparison with the control (Figure 4.11.d, e).

In PM plate 7 (Figure 4.12.a-f), further peptide nitrogen sources were evaluated. The alpha ester (compound **4a**) again retained the greatest antimicrobial efficacy in presence of these PM sources. The ether derivatives (compound **9a** and **9b**) again seemed to promote utilisation of the PM sources.

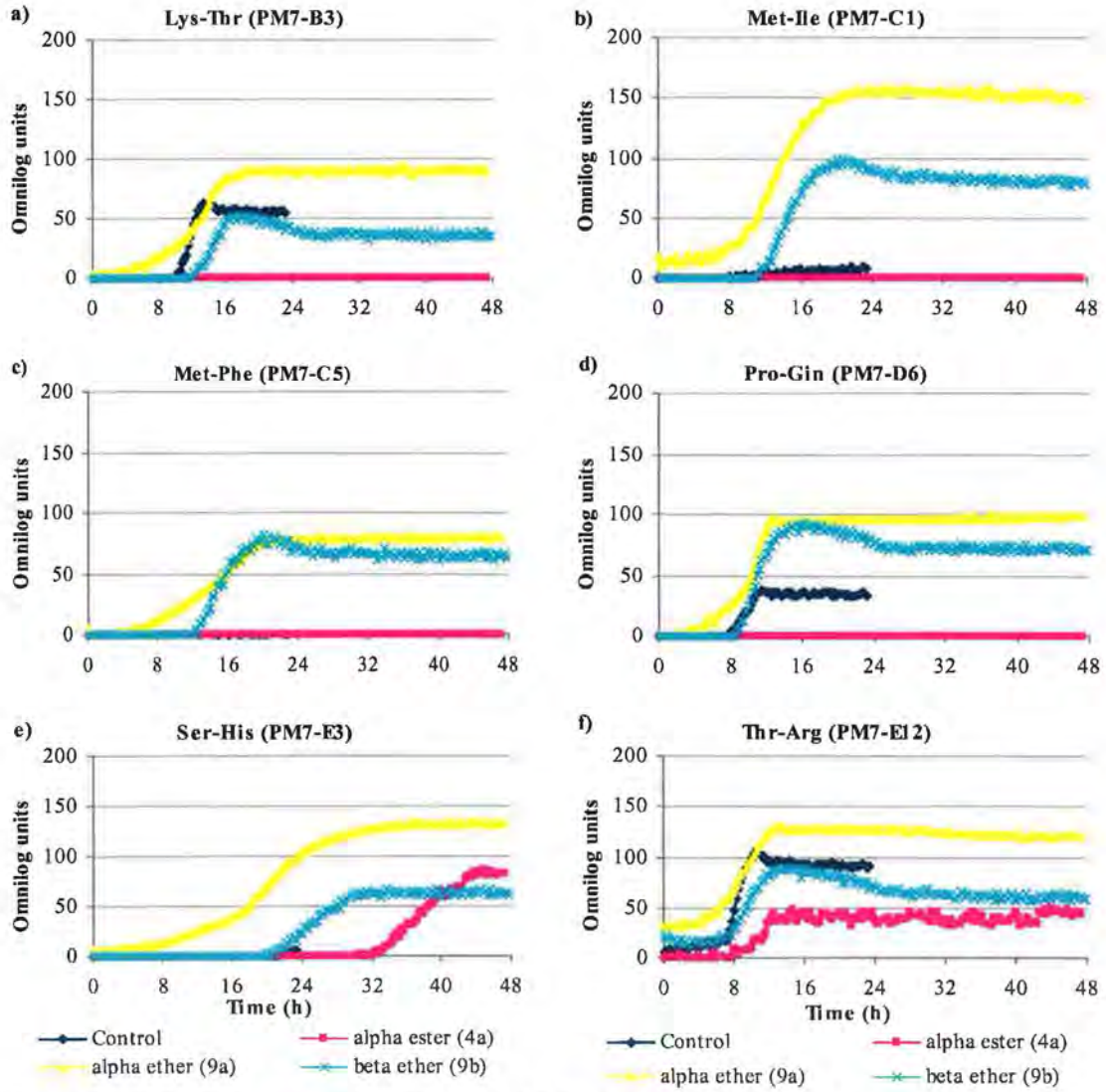


Figure 4.12. *L. monocytogenes* ATCC 7644 cells with and without CFA derivatives grown in PM7 Peptide nitrogen sources

From PM plates 1 to 8, we found the following key trends:

- In general, the alpha ester (compound **4a**) had a greater inhibitory effect on PM sources utilisation by comparison with the ethers. An exception was in the presence of a nucleoside or nucleotides, where utilisation of the PM sources was promoted in the presence of the alpha ester.
- The alpha ether (compound **9a**) generally promoted the utilisation of the PM sources above that observed for the control. Thus, the glycoconjugate bond to either an ester or an ether seems to play a role in phenotypic response to different PM sources.
- The role of anomeric configuration was again observed where the utilisation of PM sources by the beta ether (compound **9b**) was different to that of the alpha ether (compound **9a**). Generally, the beta ether (compound **9b**) promoted PM source utilisation but to a lesser extent than the alpha ether. However, the beta ether was occasionally inhibitory for up to 24 hours and most notably was particularly inhibitory in the presence of nucleotides, contrary to the trend observed for the other two CFA derivatives.

For PM plates 9 and 10, which record effects on osmolarity and pH, no particular differences or trends related to glycoconjugate linkage or anomeric configuration were observed.

Plates PM11-PM20 yielded information about how resistant/sensitive *L. monocytogenes* ATCC 7644 cells, both with and without CFA derivatives were to a variety of known inhibitors, including toxic agents and antibiotics (See Appendix 2.1 for full PM plates information).

L. monocytogenes ATCC 7644 cells treated with CFA derivatives showed different sensitivities to certain PM inhibitors compared to the control cells. For example, cells treated with alpha lauric ester (compound **4a**) became more susceptible to β-lactam antibiotics (*e.g.* cloxacillin, nafcillin, oxacillin, penicillin G) present in the PM plates, by comparison with the control cells (Figure 4.13). Beta lactam antibiotics act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. In contrast, cells treated with the CFA ether derivatives (compound **9a** and **9b**) behaved similarly to the control cells with no CFA derivatives included. Again, this is important for observing the structure/activity relationship, as it would indicate that the glycoconjugate bond might influence the mode and/or site of action of the CFA derivatives.

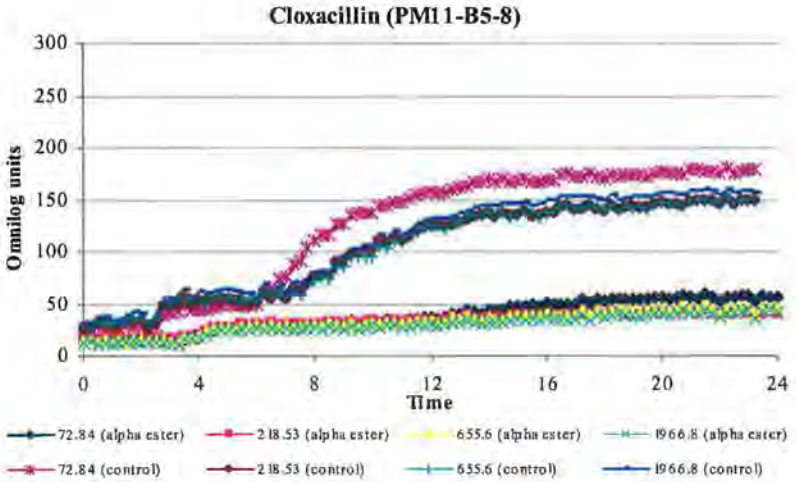


Figure 4.13 *L. monocytogenes* ATCC 7644 cells with and without alpha ester derivative (compound **4a**) grown in PM plate containing cloxacillin (β-lactam antibiotic).

Listeria control cells were susceptible to chlortetracycline at the highest concentrations tested and resistant to the two lowest concentrations (PM11, wells A5-A8). When alpha ester (compound **4a**) and alpha ether (compound **9a**) derivatives were present, *Listeria* cells displayed an increase in lag time or a decrease in growth, respectively, at the lowest concentrations tested. This suggests an additive or synergistic effect between the inhibitors, where the sensitivity to either the antibiotic at the lower concentration or the CFA derivative was increased (Figure 4.14).

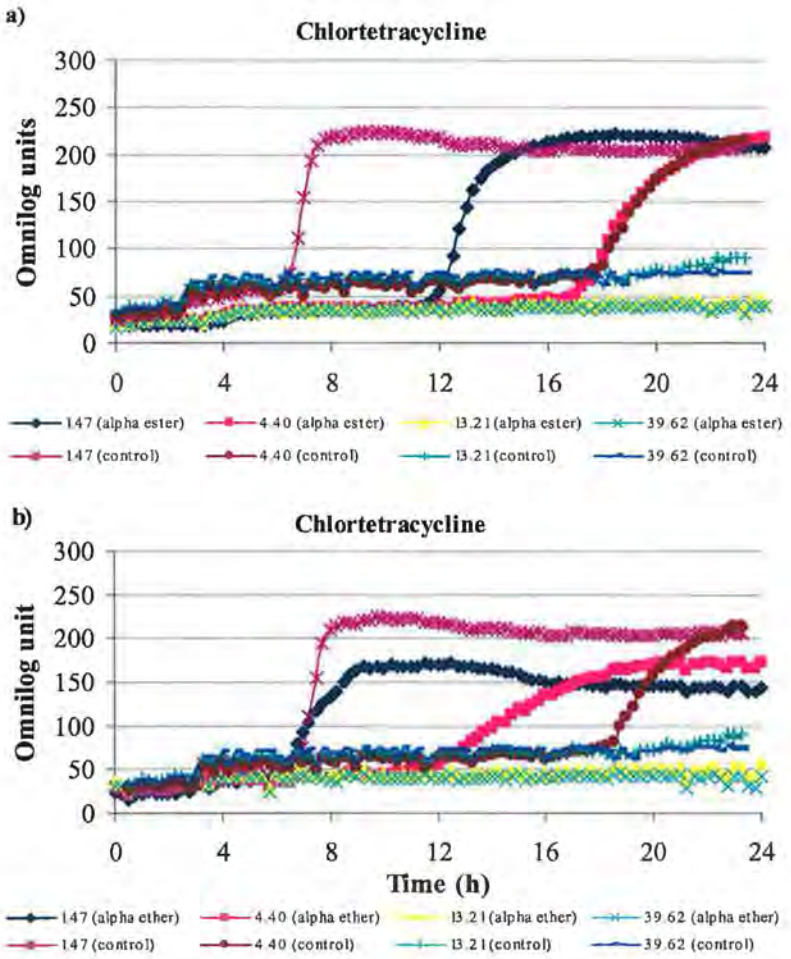


Figure 4.14 *L. monocytogenes* ATCC 7644 cells with and without a) alpha ester (**4a**) and b) alpha ether (**9a**) derivatives, grown in PM plate containing chlortetracycline.

Listeria control cells were resistant to the aminoglycosides, tobramycin, paromycin and sisomycin, at the concentrations tested, which work by binding to a site on the bacterial 30S and 50S ribosome subunits, preventing formation of the 70S complex. When the alpha ester (compound **4a**) was present, an increase in sensitivity to this class of antibiotics was observed for the two highest concentrations tested (Figure 4.15).

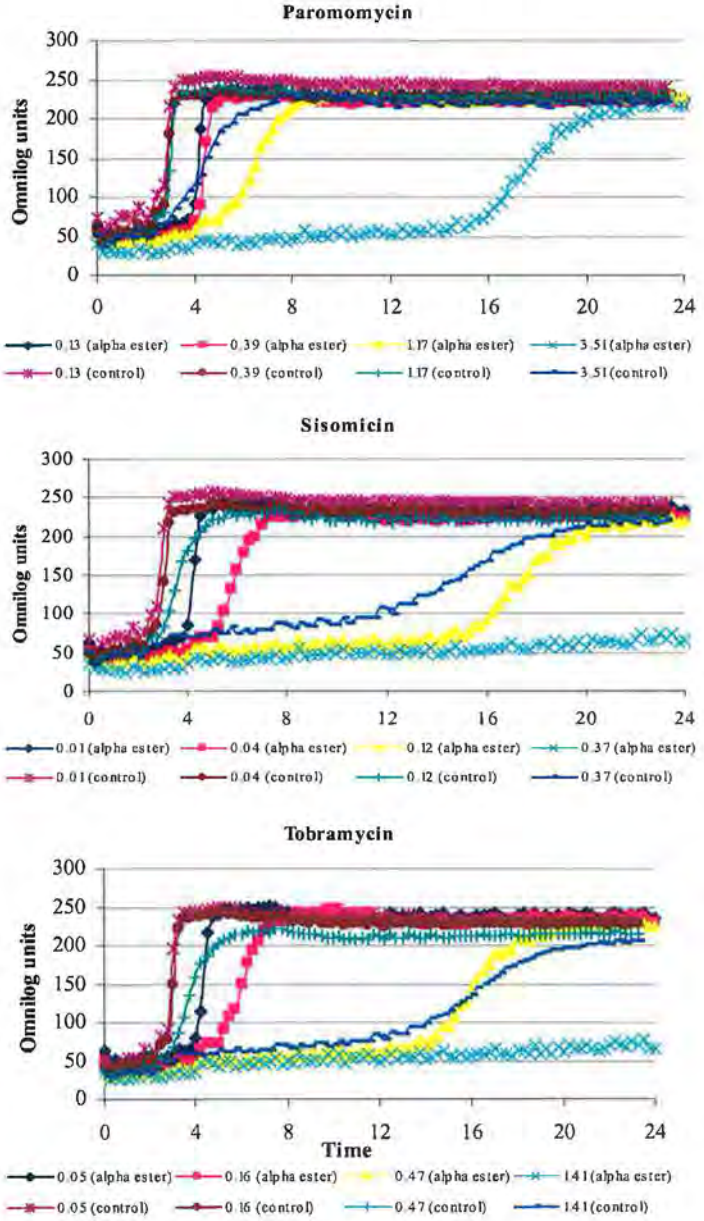


Figure 4.15 *L. monocytogenes* ATCC 7644 cells with or without alpha ester derivative (compound **4a**) grown in PM plates containing aminoglycosides.

An interesting trend was observed, where the control cells had resistance to lauryl sulfobetaine, a zwitterionic surfactant, especially at lower concentrations. In the presence of all CFA derivatives tested, *Listeria* became more susceptible to the lower concentrations (Figure 4.16) suggesting a synergy or additive effect.

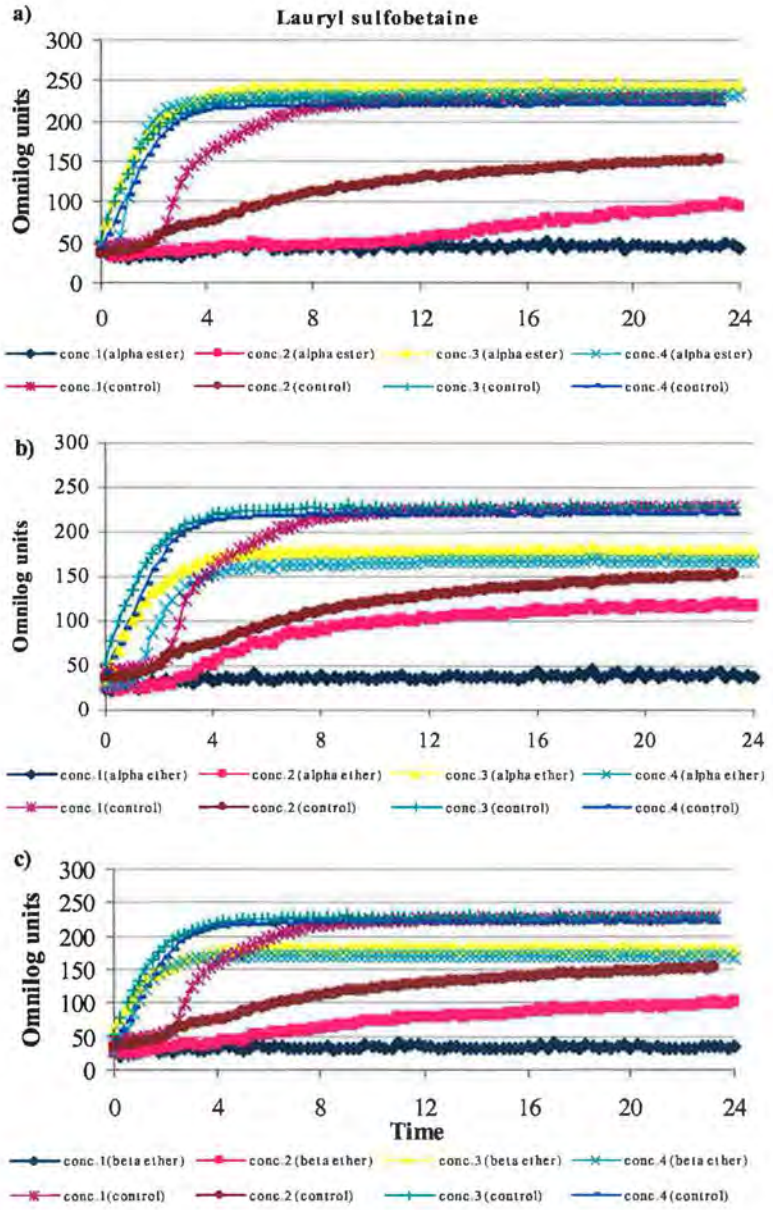


Figure 4.16 *L. monocytogenes* ATCC 7644 cells, with or without a) alpha ester (**4a**); b) alpha ether (**9a**) and c) beta ether (**9b**), grown in PM plates containing lauryl sulfobetaine.

The PM inhibitors in plates PM11 to 20 were grouped according to their mode of action. The response of *Listeria* cells treated with the CFA derivatives, to each of the PM inhibitors, was allocated a score corresponding to the response of the control cells without CFA derivative. This was related to whether there was a change (yellow colour) or not (blank) and if that change was positive or negative for the sensitivity or resistance of the cells towards the PM inhibitors. Table 4.2 summarises the key trends for the CFA derivatives tested, according to the interaction observed with the PM inhibitors and their mode of action. Statistical analysis showed a relationship/association ($p \geq 0.05$) between alpha ester (compound **4a**) and alpha ether (compound **9a**) when analysing the phenotypic responses against the antimicrobial compounds in the PM10-20. No association between alpha ester (compound **4a**) and beta ether (compound **9b**) was found. However, there was a high correlation ($p \geq 0.05$) between alpha ether (compound **9a**) and beta ether (compound **9b**). Other types of toxic compounds were not associated with any key discernible effects in conjunction with the CFA derivatives.

Table 4.2 PM inhibitors and CFA derivatives mode of action relationships.

PM inhibitors	Mode of action	α ester (4a)	α ether (9a)	β ether (9b)
Atropine	acetylcholine receptor, antagonist	+	+	+
Thiosalicylate	anti-capsule		+	
Ketoprofen	anti-capsule	-	-	
Sodium salicylate	anti-capsule		+	
Sanguinarine	ATPase, Na+/K+ and Mg++			+
Propanolol	beta-adrenergic blocker	+	+	+
Compound48/80	cell cycle modulation, Calmodulin inhibitor (gn) phospholipase C, ADP ribosylation		+	
Trifluoperazine	cell cycle modulation, DNA synthesis, Ca(2+)/calmodulin dependent protein phosphorylation and lipids and phospholipids (yst, m)			+
Cephalothin	cell wall	+		
Amoxicillin	cell wall	+		
Cloxacillin	cell wall	+		
Nafcillin	cell wall	+		
Penicillin G	cell wall	+		
Carbenicillin	cell wall	+		
Oxacillin	cell wall	+		
Azocillin	cell wall	+		
Carbenicillin	cell wall	+		
Phenethicillin	cell wall	+	+	
4-Aminopyridine	channel blocker, K+		+	
5-Chloro-7-iodo-8-hydroxyquinoline	chelator, lipophilic		-	
8-Hydroxyquinoline	chelator, lipophilic	+	+	+
Orphenadrine	cholinergic antagonist	+	+	+
Pridinol	cholinergic antagonist		+	-
Promethazine	cyclic nucleotide phosphodiesterase			+
Novobiocin	DNA unwinding, gyrase (GN), topoisomerase (GP), quinolone	+		
Sulfamonomethoxine	folate antagonist		+	
Trimethoprim	folate synthesis, dihyldrofolate reductase inhibitor		-	
Chloroxylenol	fungicide	+	+	
Tolylfluanid	fungicide	+	+	+
Captan	fungicide		-	-
D-Serine	inhibits 3PGA dehydrogenase (L-serine and pantothenate synthesis)		+	
Dodine	membrane			+
Niaproof	membrane		-	-
Benzethonium chloride	membrane		+	

Table 4.2 continued.

PM inhibitors	Mode of action	α ester (4a)	α ether (9a)	β ether (9b)
Dodecyltrimethyl ammonium bromide	membrane			+
Cetylpyridinium chloride	membrane		+	+
Poly-L-lysine	membrane		-	
Lauryl sulfobetaine	membrane	+	+	+
Amitriptyline	membrane	+	+	+
1-Chloro-2,4-dinitrobenzene	oxidation	-	+	+
Iodoacetate	oxidation	-		
Paromomycin	protein synthesis	+		
Sisomicin	protein synthesis	+		+
Tobramycin	protein synthesis	+	+	+
Chlortetracycline	protein synthesis	+	+	
Tetracycline	protein synthesis			+
Penimepicycline	protein synthesis	+		+
Thiamphenicol	protein synthesis		-	
Oleandomycin	protein synthesis		-	-
Lincomycin	protein synthesis	-		
Puromycin	protein synthesis	+	+	
Iodonitro tetrazolium chloride	respiration	+	+	+
Pentachlorophenol	respiration, ionophore, H+		-	
3,5-Dinitrobenzene	respiration, ionophore, H+	+	-	
Sodium azide	respiration, uncoupler	+		
Tetrazolium violet	respiration, uncoupler		+	+
Crystal violet	respiration, uncoupler	-		+
Oxycarboxin	respiratory enzymes,carboxamide, fungicide		+	
Diamide	thiol crosslinking agent	-	+	
Potassium chromate	toxic anion		+	+
Boric acid	toxic anion			-
Sodium cyanate	toxic anion	+		
Sodium m-arsenite	toxic anion	+	+	
Sodium metasilicate	toxic anion		+	
Sodium periodate	toxic anion		-	
Sodium orthovanadate	toxic anion	-	-	-
Sodium dichromate	toxic anion			+
Cadmium chloride	toxic cation		-	-
Lithium chloride	toxic cation	+	+	
Antimony III chloride	toxic cation	+		
Phenylarsine oxide	tyrosine phosphatase inhibitor		-	
Lidocaine	voltage-gated Na+ channels antagonist	+	+	

4.2 DISCUSSION

Various hypotheses have been proposed to explain the general mechanism of action of free fatty acids and their monoglycerides on bacteria. It was speculated that the biophysical and biochemical effects of fatty acids on the molecular structure of the cytoplasmic membrane are associated with the hydrocarbon chain of added fatty acids on insertion into the phospholipid bilayer of the membrane, thus destabilizing the membrane (Kitahara *et al.*, 2004). Based on results of electron microscopic studies, monoglycerides were proposed to act as non-ionic surfactants that penetrate and become incorporated into the bacterial plasma membrane, thereby altering membrane permeability (Bergsson *et al.*, 1998, 2001). Wang and Johnson (1992) found that monolaurin, a compound closely related to CFA esters, produced marked morphological changes in *L. monocytogenes* cells.

Other authors hypothesised that short and medium chain fatty acids diffuse into bacterial cells in their undissociated form and dissociate in the protoplasm, leading to intracellular acidification with resultant inactivation of intracellular enzymes and inhibition of amino acid transport (Freese *et al.*, 1973; Viegas and Sa-Correia, 1991). For the compounds in this study, this particular explanation would stand only if we consider the liberation of lauric acid from the ester derivative by hydrolases as the sole mechanism of action, however, this then does not explain (or involve) the mode of action of the ether derivatives.

The mechanism of the induction of autolysis by these types of compounds, including sucrose esters of fatty acids, remains to be elucidated, although it is suggested that they may disorganize the membrane structure and trigger an as yet unidentified

autolysin control system, possibly located in and/or on the cell membrane (Tsuchido, *et al.*, 1993).

All of the CFA derivatives studied here, except for the beta ether (compound **9b**), had the ability to affect the cytoplasmic membrane of *S. aureus* as judged by the leakage of 260-nm-absorbing material assay and the *Bac*Light 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, which indicated no membrane damage, the beta ether (compound **9b**) had a significant 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 other than membrane damage. The BacLight Live/Dead fluorescence and the ATP luminescence assays focus on the viability of microorganisms, but from different perspectives. The first assesses the permeability of the cell membrane and the second involves the metabolic state of the cell. For example, the beta lauric ester (compound **4b**), which had a high antimicrobial activity, showed poor ability to permeabilize the membrane as measured by the Live/Dead fluorescence assay, but showed a decrease in the metabolic activity using the ATP luminescence assay. These results also suggest that the CFA derivatives have mechanisms of action not solely related to membrane damage.

It must also be noted, however, that the use of methods involving uptake of fluorescent probes for investigation of membrane integrity can give rise to misleading results, due to the ability of cells to extrude the probes or to uptake stain while retaining viability (Ueckert *et al.*, 1995; Breeuwer and Abee, 2000). The use of multiple methods

to determine diverse mechanisms of action of these derivatives is advisable, therefore further assays, such as the Phenotype MicroArray assay were considered.

All CFA derivatives had an effect on methicillin-resistant *S. aureus* to an extent similar to that of the non-resistant strains, suggesting that the mechanisms of methicillin resistance did not affect the derivatives efficacy. Although lauric acid and CFA derivatives might not be as effective compared with other antimicrobial agents (antibiotics), it may be a potential antimicrobial material and ointment base for infection control. Combinations of lauric acid or its derivatives with other antimicrobials have also shown additive or synergistic effects against pathogenic or spoilage bacteria in several food matrices (Bell and De Lacy, 1987, Wang and Johnson, 1997; Blaszyck and Holley, 1998; Yamazaki *et al.*, 2004).

Other researchers have reported that monolaurin at sub-MIC concentrations inhibited production of β-lactamases, toxic shock syndrome toxin 1 and other exoproteins in *S. aureus* at the level of transcription by interfering with signal transduction (Schlievert *et al.*,1992; Projan *et al.*, 1994). Moreover, 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).

Generally, isolates of *L. monocytogenes* are susceptible to a large range of antibiotics (Hof *et al.*, 1997; White *et al.*, 2002), however *Listeria* spp. are regarded as tolerant to all β-lactam antibiotics (Hof, 2003). The CesRK two-component system contributes to the intrinsic resistance of *L. monocytogenes* LO28 to antibiotics of the β-lactam family (Kallipolitis *et al.*, 2003; Gottschalk *et al.*, 2008). The PM results confirmed that *L. monocytogenes* ATCC 7644 had resistance against β-lactam

antibiotics, but on exposure to the alpha ester (compound **4a**) became more susceptible to the antibiotics. This suggests that similar to monolaurin, the alpha ester derivative (compound **4a**), at sub-MIC concentrations, could also inhibit production of β-lactamases in *L. monocytogenes* at the level of transcription by interfering with signal transduction, as proposed by Schlievert *et al.* (1992); Projan *et al.* (1994) and Ruzin and Novick (1998).

It is also possible that fatty acid derivatives might 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 against 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 *S. aureus*.

The phenotypic responses of *Listeria* cells with and without CFA derivatives provided very useful information confirming that glycoconjugate bond and anomeric configuration had an effect on the antimicrobial efficacy and mode or site of action of the CFA derivatives. When considering the role of the glycoconjugate bond, the alpha ester (compound **4a**) proved to be the most inhibitory compound, by comparison with the ether (compound **9a**). Additionally, the alpha configuration was the most inhibitory when assessing the anomeric configuration. Statistical analysis showed an association ($p \geq 0.05$) between alpha ester (compound **4a**) and alpha ether (compound **9a**) when analysing the phenotypic responses against the antimicrobial compounds in the PM10-20, as well as between alpha ether (compound **9a**) and beta ether (compound **9b**). No

association between alpha ester (compound **4a**) and beta ether (compound **9b**) was found.

Nucleotides play central roles in metabolism. In that capacity, they serve as sources of chemical energy (adenosine triphosphate and guanosine triphosphate), participate in cellular signaling (cyclic guanosine monophosphate and cyclic adenosine monophosphate), and are incorporated into important cofactors of enzymatic reactions (coenzyme A, flavin adenine dinucleotide, flavin mononucleotide, and nicotinamide adenine dinucleotide phosphate). Cyclic adenosine monophosphate (cAMP, cyclic AMP or 3'-5'-cyclic adenosine monophosphate) is a secondary messenger that is important in many biological processes. cAMP is derived from adenosine triphosphate (ATP) and used for intracellular signal transduction in many different organisms, conveying the cAMP dependent pathway. The increased response of *Listeria* treated with alpha ether (compound **9a**) and alpha ester (compound **4a**) in the presence of nucleotides as PM sources, suggests that these compounds could have an effect on signal transduction.

The beta ether derivative (compound **9b**) inhibited the utilisation of N-acetyl-D-glucosamine (NAG) as a PM source in contrast to the utilisation observed in the control assay, as well as that in the presence of the alpha ester (compound **4a**) and alpha ether (compound **9a**). NAG is a key component of peptidoglycan, which is an essential component of the bacterial cell's wall. The bacterial growth inhibition could be attributed to the depletion of peptidoglycan content. Our findings are concurrent with the studies by Ved *et al.* (1984c), who reported that dodecylglycerol, the 12-carbon alkyl ether of glycerol, inhibited the synthesis of the peptidoglycan *of Streptococcus faecium* ATCC 9790 and *Streptococcus mutans* BHT. That metabolic regulation represents the second known mode by which dodecylglycerol expresses antibacterial activity. The first one

was reported by the same authors, Ved *et al.*, (1984a,b), who found that dodecylglycerol stimulated autolysin activity, which degraded cell-wall peptidoglycan. However, they did not conclude that those were the only modes of action of dodecylglycerol, as other mechanisms could be also possible.

5. SCREENING OF OTHER NATURAL ANTIMICROBIAL COMPOUNDS (ESSENTIAL OIL COMPONENTS AND NISIN)

The present study was designed to establish the MICs of selected plant essential oil (EO) individual components against food-borne pathogens, focusing on *Listeria monocytogenes* and a range of spoilage bacteria. Additionally, the MIC of nisin was determined to investigate potential synergistic activity with the optimum EO components. This screening of natural compounds like nisin and essential oil components for antimicrobial activity was performed to evaluate the potential for combination strategies with the CFA derivatives. Generally, nisin is more active at lower pH values; therefore the combined effect between nisin and the EO components at neutral pH and lower pH levels was investigated. Furthermore, the efficacy of the combinations (additive, synergistic, or antagonistic) on the inhibition of the pathogens was evaluated.

5.1 RESULTS

5.1.1 Antimicrobial activity of EO components and nisin

The antimicrobial activity of 10 plant essential oil individual components (allylanisole, camphor, carvacrol, caryophyllene, citral, eucalyptol, linalool, sabinene, terpinenol and thymol) and nisin was evaluated against a range of foodborne pathogens and spoilage bacteria. The MICs of all the antimicrobials were calculated and are presented in Table 5.1.

Nisin and EO components showed varying degrees of individual activity against the microorganisms tested. Nisin displayed a very low antibacterial activity against the Gram-negative bacteria, although this was expected. Among the EO components, carvacrol and thymol displayed the highest antimicrobial activity against the full range of bacteria with MICs ranging from 1.25 to 2.5 mM. The MIC for carvacrol against *Pseudomonas* spp. was 5 mM. Citral had a high antimicrobial activity against *Listeria* spp. with MIC values between 2.5 and 5 mM. Citral, sabinene and terpinenol showed moderate activity against *E. coli* ATCC 25922 with MIC values of 12.5 mM. In general, Gram-positive bacteria were more sensitive to all the EO components than Gram-negative bacteria. *L. monocytogenes* was one of the most sensitive organisms, whereas *Pseudomonas* spp. were the most resistant microorganisms.

Some of the EO components tested, like camphor and sabinene, had a negligible antimicrobial activity possibly due to poor solubility in water.

Table 5.1 Minimum Inhibitory Concentration (MIC) values of nisin (μg/mL) and EO individual components (mM) at pH7.

Nisin or EO components / Microorganism	Nisin	Allylanisole	Camphor	Carvacrol	Caryo-phyllene	Citral	Eucalyptol	Linalool	Sabinene	Terpinenol	Thymol
Lb. sakei ATCC15521	9.77	>25	>25	2.5	3.13	10	>20	20	≥25	>10	2.5
L. innocua NCTC11288	320.0	>25	12.5	1.25	25	5	>10	>10	>25	>5	1.25
L. monocytogenes ATCC7644	249.2	25	12.5	1.25	>25	2.5	>10	25	>25	12.5	1.25
L. monocytogenes NCTC11994	225.0	25	>25	2.5	25	2.5	>10	25	≥25	12.5	1.25
L. monocytogenes NCTC7973	266.7	25	12.5	1.25	>25	2.5	>10	≥25	>25	>10	1.25
E. coli ATCC25922	ND	>50	25	2.5	>50	12.5	>25	25	12.5	12.5	1.25
P. fluorescens	ND	>50	50	5	>50	25	>25	>50	50	50	2.5
P. putida	ND	>50	50	5	>50	>25	>25	>50	50	50	1.25

For each analysis the MIC was recorded as the concentration that resulted in total inhibition of all replicates after 24 hours. ND: Not determined.

5.1.2 Influence of pH on antimicrobial activity.

In order to investigate the influence of pH on the antimicrobial activity of Nisin and EO components on *Listeria* strains, experiments were performed in media adjusted to pH 7, 6 and 5.5. Bacterial growth was supported at all pH levels tested. A decrease in the pH from neutral to pH 6 or pH 5.5 resulted in enhanced activity for nisin, with an approximate 2-fold decrease in MIC values recorded (Figure 5.1).

A lack of useful interactive effect with pH was observed for the EO components, carvacrol, citral and thymol, with no considerable reduction in MIC values.

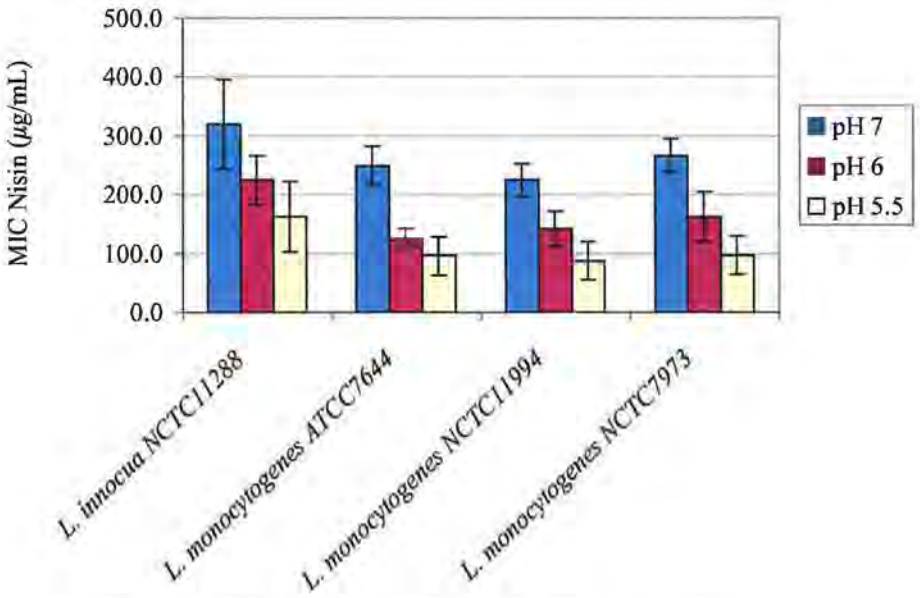


Figure 5.1 Influence of pH on MIC_{Nisin} (μg/mL) values

5.1.3 Synergy studies between EO components and nisin

The quantitative effects of selected EO components (carvacrol, citral and thymol) in combination with nisin against *Listeria* strains were described in terms of FIC indices (Table 5.2). At pH 7 none of the combinations tested displayed synergistic activity against the bacteria strains used in this study, however, combinations of EO components with nisin showed additive effects, and at pH 6 an enhanced additive effect was generally observed with Carvacrol or Citral in combination with nisin. At pH 6, citral combined with nisin had synergistic effects against *L. monocytogenes* ATCC7644. No antagonism was observed for any of the combinations evaluated.

Table 5.2 FIC indices for combinations of nisin and selected EO components against *Listeria* species at different pHs.

Combination	pH	*L. innocua* NCTC11288	*L. monocytogenes* ATCC7644	*L. monocytogenes* NCTC11994	*L. monocytogenes* NCTC7973
Nisin +	7	1.16 ± 0.28 (A)	1.14 ± 0.45 (A)	1.23 ± 0.40 (A)	0.72 ± 0.21 (A)
Carvacrol	6	1.19 ± 0.31 (A)	0.95 ± 0.29 (A)	1.08 ± 0.33 (A)	1.08 ± 0.38 (A)
Nisin +	7	1.09 ± 0.31 (A)	0.91 ± 0.40 (A)	0.77 ± 0.49 (A)	1.17 ± 0.29 (A)
Thymol	6	1.02 ± 0.35 (A)	0.94 ± 0.35 (A)	1.06 ± 0.35 (A)	1.25 ± 0.25 (A)
Nisin +	7	1.00 ± 0.20 (A)	0.92 ± 0.14 (A)	1.31 ± 0.38 (A)	1.25 ± 0.35 (A)
Citral	6	1.00 ± 0.25 (A)	0.5 ± 0.00 (S)	0.63 ± 0.18 (A)	0.75 ± 0.00 (A)

(S): Synergy (FIC < 0.5), (A): Addition (0.5 ≤FIC ≤4) or (An): Antagonism (FIC > 4). Standard deviations are indicated beside each value.

5.2 DISCUSSION

Listeria monocytogenes is currently a worldwide concern affecting numerous countries. In the European Union (EU), in addition to the economic consequences and threats associated with outbreaks, listeriosis remains of great public health concern, as it has one of the highest case fatality rates of all the foodborne infections: 20-30% (De Valk *et al.*, 2005). International guidelines for microbiological criteria in respect of many foodstuffs have not yet been established. In USA there is a 'zero tolerance' (no detectable level permitted) for *L. monocytogenes*, whereas current EU legislation states products must either comply with a limit of 100 colony forming units per gram (cfu/g) throughout their shelf-life or have an absence of *L. monocytogenes* in 25g for ready-to-eat (RTE) foods intended for infants and for special medical purposes (Regulation (EC) No 2073/2005). This study aimed to investigate the antimicrobial activity of the combination of nisin, a bacteriocin, with selected EO components focusing on *Listeria monocytogenes*. Investigating combination strategies can lead to a reduction in the concentrations required for pathogen or spoilage microorganism control, which can be particularly important in the case of antimicrobial or preservative properties for foodstuffs due to the potentially high sensory impact.

Essential oils or their individual components, *e.g.*, carvacrol, thymol, eugenol and citral, have been shown to exhibit antimicrobial activity against foodborne pathogenic bacteria (*Escherichia coli* O157:H7, *Salmonella* spp. *Listeria* spp., *Bacillus cereus* and *B. subtilis*), and food spoilage bacteria (Helander *et al.*, 1998; Dorman and Deans, 2000; Olasupo *et al.*, 2004; Friedman *et al.*, 2004), therefore they have potential for application as novel food preservatives.

The observation in this study of the potential of EO components, singly and in combination with bacteriocins, to inhibit foodborne and spoilage bacteria, is a further indication of the antimicrobial activity of these compounds.

Synergistic effects are important in the light of the potential for combination strategies between bacteriocins and EO components as well as CFA derivatives with other antimicrobials for practical application to microbiological issues within the food and other industries. Preuss *et al.*(2005), found that *Origanum* oil, carvacrol, and other essential oils, combined with monolaurin killed *S. 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.

Previous studies have concentrated more on the antimicrobial activity of different plant EO's and some of their individual components, but a limited number of studies have focused on the potential combination of these compounds with other antimicrobials. Most of the synergistic studies were tested using *Bacillus* spp. Pol and Smid (1999) have reported a synergistic effect between nisin and carvacrol against *B. cereus* and *L. monocytogenes*. Additionally, Ettayebi *et al.* (2000) observed that the concentration of nisin required for antimicrobial activity against *L. monocytogenes* and *B. subtilis* could be lowered by combination with thymol. In the current work, combining thymol with nisin did not yield synergistic activity, however the combination was additive but somewhat influenced by pH, with enhanced additive activity at pH 7 instead of pH 6. Periago and Moezelaar (2001) also reported synergistic action between carvacrol and nisin against *B. cereus*. All the mentioned studies have reported synergistic effects on viable counts, although true synergistic effect can be best confirmed by the use of FIC indices as suggested by Olasupo *et al.* (2004).

The combination of bacteriocins along with other natural preservatives might increase their efficacy within food matrices against some Gram-negative spoilage bacteria known to show resistance to antimicrobials, such as *Pseudomonas* spp. (Hammer *et al.*, 1999; Holley and Patel, 2005). Also, the use of high concentrations of nisin may encourage the selective growth of nisin-resistant bacterial sub-populations (Crandall and Montville, 1998). Thus, the combination of EO components with bacteriocins could lead to useful efficacy against both spoilage and pathogenic target organisms and reduce the concentration of bacteriocin required reducing the risk of resistance.

In general, the Gram-positive bacteria were more sensitive to nisin than the Gram-negative, and in particular, the *Listeria* spp. were the most susceptible organisms. Plant EO components and nisin are generally more active against gram-positive bacteria than gram-negative bacteria (Cleveland *et al.*, 2001; Burt 2004). Davidson and Branen (2005) suggested that the outer membrane surrounding the cell wall of Gram-negative bacteria might restrict diffusion of hydrophobic compounds through its lipopolysaccharide covering.

When EO components are used in combination with other antimicrobials, such as bacteriocins, the dose of added phenolic compounds could be lowered thereby decreasing their impact on the food flavour and taste (Galvez *et al.*, 2007).

The effectiveness of nisin increases when pH decreases due to the higher solubility and stability of nisin at lower pH (Lui and Hansen, 1990).

According to Juven *et* al. (1994), the increased antibacterial activity of an essential oil at low pH can be related to the fact that the essential oil constituents become more hydrophobic at low pH and dissolve better in the lipid phase of the bacterial membrane.

In the present study no significant effect was observed in the efficacy of the EO components tested at lower pH levels. However, in the case of nisin combined with citral, a synergistic effect was observed when pH was lowered from neutral to pH 6.

Future direction for this work could include synergy studies between EO components and CFA derivatives. As well as the use of EO components as scaffolds for further derivatisation or synthetic elaboration including analogue synthesis. The potential advantages of EO component derivatisation could include improved acceptability of the organoleptic profile and/or enhanced antimicrobial efficacy resulting from a structure/ activity relationship studies of closely related synthetic analogues.

Chapter 6 OVERALL DISCUSSION

Despite several decades of improved antimicrobial therapy, infectious diseases caused by bacteria, fungi, viruses and parasites are still a major cause of morbidity and mortality in humans (Cos *et al.*, 2006). The emergence of drug resistance in bacteria, *e.g.* methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), extended spectrum β-lactamase-producing Enterobacteriaceae and multi-drug resistant *Mycobacterium tuberculosis* (MDRTB), continues to evolve and spread globally. This is posing a threat to human health, since the number of empirical agents suitable for selected indications is reduced and the pace at which new antibiotics are being produced is slowing (Russell, 2002; Isturiz, 2008).

Simultaneously, the epidemiology of foodborne diseases is rapidly changing as newly recognized pathogens emerge and well recognized pathogens increase in prevalence or virulence and become associated with new food vehicles.

Consumer awareness and 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 for use in foods.

Due to their chemical diversity, natural products, either as pure compounds, their derivatives or as extracts, may provide opportunities for new drug leads or nature-derived preservatives that can be safely incorporated into food products (Cos *et al.*, 2006).

In this present study, the effect of polyhydroxylated fatty acid derivatives as inhibitors of a range of Gram positive and Gram negative microorganisms of concern to the food and healthcare industries has been examined.

Carbohydrate fatty acid derivatives are an interesting class of compounds with appealing characteristics and many and diverse applications. The effects of the fatty acid chain length, the hydrophilic core, as well as the degree of substitution on the carbohydrate are all factors that will affect the properties of these compounds and, as a result, their applications.

The literature has many reports of novel antimicrobial CFAs but it is difficult to compare them. Deductions from the literature are difficult for a number of reasons: the lack of studies using pure compounds, lack of comparative studies, use of different test organisms, use of different test conditions, etc. In the present study, using an iterative design approach to generate a novel set of pure compounds, in tandem with carefully designed evaluations, we have been able to eliminate as much of the uncertainty with respect to the issues outlined above as possible. Thus, allowing antimicrobial efficacy structure/activity relationship comparisons.

Ester and ether derivatives of the same carbohydrate fatty acid have been found to be more effective than the free fatty acid, but differences between antimicrobial efficacy have been observed which suggested that the nature of the bond conjugating the fatty acid to the carbohydrate and the carbohydrate in itself might play a role in antimicrobial activity.

Although the antimicrobial effects of fatty acid derivatives are well established, the mechanism of action of such compounds is poorly understood. Furthermore, in contrast to the extended investigation on mode of action of monoglycerides, there are few studies that attempt such an insight for other fatty acid derivatives. Research on the antimicrobial action of fatty acid derivatives has focused on their effects on cellular membranes (Kabara and Marshall, 2005). However, the *proof* that this is the sole mechanism is not extensive. Moreover, this explanation does not account for structural differences between compounds.

Based on leakage of components at 260nm, it was clearly demonstrated that carbohydrate fatty acid derivatives **4a-b** and **9a** (alpha ester, alpha ether and beta ester), disrupted the cell membrane, causing an increase in substances absorbing at 260nm. In accordance, permeabilization by the fluorescent nucleic acid stains, SYTO9 (stains all cells green) and propidium iodide (stains cells with damaged membrane red) using the BacLight assay with the same compounds, was also found to disrupt the outer membrane of *S. aureus* at MIC levels. Nevertheless, compound **9b** (β ether) notably had a significant effect on bacterial growth as seen by the increase in lag time and decrease of ATP levels, suggesting that this compound could have a mechanism/site of action other than membrane damage. The different phenotypical responses of *Listeria monocytogenes* to the CFA derivatives also support the proposed alternative mechanism/site of action of ester and ether derivatives.

Potential combination strategies of naturally derived compounds are of great interest to both food and health-care industries, because of their broad spectral range, lower toxicity and cost (Lambert and Lambert, 2003). Therefore other natural antimicrobials namely essential oil individual components and nisin were also included in this study to assess other structures and combination potential. Conditions of the media, such as lowering the pH, was shown to enhance the efficacy of some compounds, hence this should be taken into account in addition to other extrinsic factors for their application in food.

7. CONCLUSIONS

This study has developed and used a regio-selective synthesis for carbohydrate fatty acid derivatives, which yielded pure compounds for evaluation as antimicrobials as a key objective of our work. The emphasis on compound purity and defined structures was critical to discern the impact of structural variations on antimicrobial efficacy, and that activity could be related to the structural changes with confidence.

In summary, this study draws the following conclusions:

(i) When assessing the variation of fatty acid chain length, lauric acid derivatives were found to be more active against Gram positive bacteria by comparison to caprylic acid derivatives.

(ii) In general, the CFA derivatives were found to be significantly more active against the Gram positive bacteria than the Gram negative bacteria tested.

(iii) Lauric esters of α- and β-Methyl glucopyranoside and α-Methyl mannopyranoside as well as the lauric ether of α-Methyl glucopyranoside were the most active compounds, comparable to monolaurin for antimicrobial efficacy.

(iv) 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 and the anomeric position of the carbohydrate are involved in the antimicrobial activity of the fatty acid derivatives and that the nature of the glycoside bond also has a significant effect on efficacy.

(v) When assessing the role of carbohydrate versus non-carbohydrate hydrophilic cores (carbohydrate and pentaerythritol laurates), it was found that the nature of the carbohydrate core plays a role in the efficacy of carbohydrate fatty acid derivatives as antimicrobials, and therefore further optimisation may be possible.

(vi) Monoester derivatives were more effective than di-ester derivatives, due to poor solubility of the di-ester compounds.

(vii) Membrane damage was found to be one of the mechanisms/site of action for these types of compounds.

(viii) The results for the beta ether derivative **9b** indicate an alternative mechanism or site of action.

(ix) Carbohydrate fatty acid derivatives have potential as effective antimicrobial compounds for use as preservatives to address a range of microbiological stability and safety issues.

(x) Useful combination with other antimicrobials (antibiotic or natural inhibitors) may provide opportunities for new drug leads suitable against drug resistant bacteria.

8. FUTURE RECOMMENDATIONS

The use of a synthetic route to control production of regio-chemically defined compounds allowed the optimization of the carbohydrate moiety configuration and glycoconjugate linkage with regard to antimicrobial efficacy.

The quantity yield of the test compounds was at the expense of purity where the yield was relatively low, minimising the number of bacteria that could be studied. However, to confirm the trends outlined with respect to the importance of the carbohydrate moiety and the role of the nature of the glycoconjugate bond, further studies are warranted using a wider range of bacterial species and strains, in particular Gram-positive microorganisms. This would allow for evaluation of potential species and strain effects and an expanded trial incorporating a wide range of *S. aureus* strains is warranted. This study should also incorporate the potential for stress response effects or potential for bacterial resistance or adaptation to the novel compounds.

This study has indicated that α-monosaccharide mono-fatty acid esters and their corresponding ethers have most potential and future studies are proposed to develop an alternative enzymatic synthesis protocol to yield greater quantities of selected compounds thereby allowing full screening studies using a more limited number of test compounds but with a full array of bacteria.

An alternative approach, using more advanced analytical techniques is proposed to monitor the degradation of CFA derivatives in cell suspensions. Techniques such as LC-NMR (Liquid Chromatography - Nuclear Magnetic Resonance) and LC-MS (Liquid Chromatography - Mass Spectrometry) are recommended.

Carbohydrate fatty acid derivatives have potential as effective antimicrobial compounds for use as preservatives to address a range of microbiological stability and safety issues, therefore, studies incorporating the CFA derivatives to different food systems are advisable to assess the effect of food components on the CFA derivatives efficacy.

Combining CFA derivatives with EO's or their individual components may be useful, as CFA derivatives could help the use and incorporation of EO's in different systems overcoming solubility issues and decreasing the organoleptical effect of EO's in certain food products.

More studies are advisable to assess the combination effects of the CFA derivatives with other antimicrobials, like antibiotics, which may provide opportunities for new drug leads suitable against drug resistant strains.

Further studies on the mode of action of the ether derivatives are required. Electron microscopy assays might be valuable to assess the effect of the ether derivatives on the bacterial cell envelope. Studies assessing the possibility of the ether derivatives to bind to cell surface receptors and interfere with signal transduction are also required.

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APPENDICES

Appendix 1

1.1 Peer reviewed publications

1.2 Congress & Conferences

Appendix 2

2.1 Phenotype MicroArray plates list

2.2 PM kinetic curves

2.2.1 Alpha ester (4a)

2.2.2 Alpha ether (9a)

2.2.3 Beta ether (9b)

2.3 PM hits plates 1-10

2.4 PM hits plates 11-20

2.4.1 Alpha ester (4a)

2.4.2 Alpha ether (9a)

2.4.3 Beta ether (9b)

APPENDIX 1

1.1 Peer reviewed publications

Nobmann, P.; Dunne, J.; Henehan, G. and Bourke, P. Antimicrobial activity and mode of action of novel carbohydrate fatty acid derivatives against *S. aureus* and methicillin resistant *S. aureus* (MRSA). *Journal of Applied Microbiology* ("Accepted Article"; October 28th 2009; doi: 10.1111/j.1365-2672.2009.04622.x)

Nobmann, P.; Smith, A.; Dunne, J.; Henehan, G. and Bourke, P. (2009) The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. *International Journal of Food Microbiology* **128** (3), 440-445

Smith, A.; Nobmann, P.; Henehan, G.; Bourke, P. and Dunne, J. (2008) Synthesis and antimicrobial evaluation of carbohydrate and polyhydroxylated non-carbohydrate fatty acid ester and ether derivatives. *Carbohydrate Research* **343** (15), 2557-2566

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ORIGINAL ARTICLE

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

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Keywords
antimicrobial activity, carbohydrate fatty acid derivatives, membrane damage, mode of action, monolaurin, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus*.

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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 (MICs) and the effect of CFA derivatives on lag phase were determined using a broth microdilution method. Lauric acid carbohydrate esters and corresponding ether analogues showed the greatest antimicrobial activity with MIC values between 0·04 and 0·16 mmol l^{-1}. Leakage studies at 260 nm following exposure to CFA derivatives at 4× MIC showed a significant increase in membrane permeability for all compounds, after *c.* 15 min 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 the 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.

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. *Staphylococcus 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). *Staphylococcus aureus* is also one of the most common causes of bloodstream infection (or bacteremia) (Petti and Fowler 2003).

Methicillin-resistant *Staph. aureus* (MRSA) and antibiotic resistance is a global concern. *Staphylococcus aureus* becomes methicillin resistant by the acquisition of the *mecA* gene that 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 MRSA are increasing in both hospital and community settings (Chopra 2003). In Ireland, there were 1394 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 (CFA) esters are biodegradable, nontoxic compounds currently being used as nonionic 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 nonspecific and development of resistance to these compounds has not been reported (Kabara and Marshall 2005). Kitahara *et al.* (2006) proposed lauric acid (LA) 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 LA, acted synergistically with penicillin G at concentrations below its minimum micelle concentration. Kitahara *et al.* 2006 found that a LA 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 ML, a compound closely related to CFA esters, produced marked morphological changes in *Listeria 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 ML at subminimum inhibitory concentrations (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, ML 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 LA ($t_{1/2}$ of *c.* 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 because of liberation of LA 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 analogue 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 CFA 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 260 nm, BacLight Live/Dead assay and cell viability using BacTiter Glo assay.

Materials and methods

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, Barcelona, 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.

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

Test compounds

LA, caprylic acid (CA), and their corresponding monoglycerides, ML and monocaprylin (MC) (Sigma-Aldrich; *c.* 99% purity), were used as standards for comparison against the CFA derivatives. Nisin, from *Lactococcus lactis* 2·5% (≥1000 000 IU^{-1} g^{-1}), was purchased from Sigma, and was used as a positive control compound for membrane disruption studies. CFA derivatives used in this study (Fig. 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.

Minimal inhibitory concentration

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 100 μl in 96-well microtiter plates (Sarstedt Ltd, Drinagh, Co. Wexford, Ireland). Each well was inoculated with 100 μl of the test organism in TSB to a final concentration of *c.* 1×10^6 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: (i) uninoculated media without test compound to assess changes in the media; (ii) uninoculated media containing the test compound to assess background noise; (iii) inoculated media without test compound to evaluate the microbial growth under optimal conditions; (iv) 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.

Increase in lag time ($\Delta\lambda$)

The increase in lag time was calculated using data from absorbance-based broth microdilution assays using Gen5™ software (BioTek, Bedfordshire, UK). The $\Delta\lambda$ 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}.

Effect of antimicrobial compounds on the cell membrane

For the mode of action studies, the laurate glucopyranoside derivatives (compounds **1–4**) were selected. These are a closely related group of compounds that 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.

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 *c.* 10^9 CFU ml^{-1}. The CFA derivatives were added to bacterial suspensions at 4× 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 min; centrifuged at 10 000 *g* for 10 min at 4°C. Two hundred microlitres of supernatant for each treatment was added to the wells of a

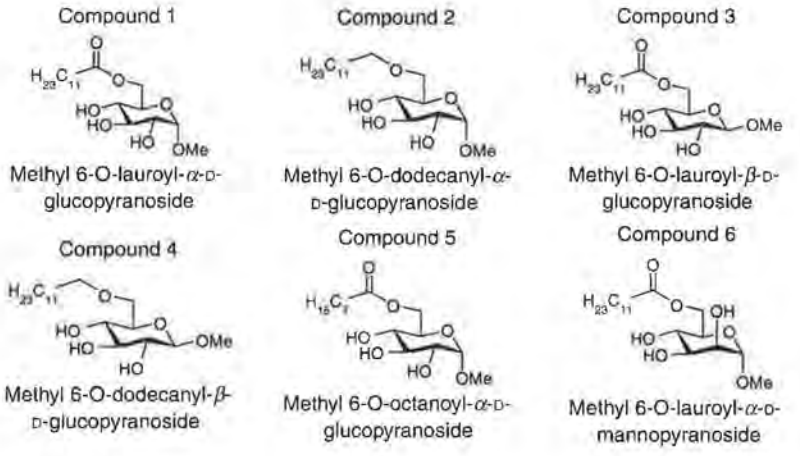

Figure 1 Structures of the novel carbohydrate fatty acid derivatives investigated for antimicrobial activity.

96-well plate (UV–transparent flat-bottom microplates, Corning-Costar Cat. No. 3635; Fisher Scientific, Ireland), 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: ML. Where applicable, independent readings were also taken, in the presence of antibacterial agents only, to enable corrections for background contributions.

Modified Live/Dead BacLight assay

The Live/Dead® BacLight viability kit from Molecular Probes, Inc. (Eugene, OR, USA) 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. Twenty-five millilitres of the bacterial culture was concentrated by centrifugation at 10 000 *g* for 10 min. 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 4× 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 1 h. 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 per ml.

A volume of 100 μl of each bacterial or treatment suspension was added in triplicate into separate wells of a 96-well microplate (black; Nunc). A 1× stain solution was prepared by mixing component A (3·34 mmol l^{-1} SYTO 9 dye) and component B (20 mmol l^{-1} propidium iodide) in equal proportion. To each well, 100 μl of 1× stain solution was then added, 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 centred 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 *vs* percentage of live cells in the *Staph. aureus* suspension.

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 4× MIC. Each well of a 96-well microtiter plate contained 100 μl of the inoculum and 100 μl 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 h. One hundred microlitres 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 5 min, and luminescence was recorded in a multi-detection microplate reader (Synergy HT).

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 (least square differences) testing at $P < 0{\cdot}05$ level using SPSS software, ver. 15 (SPSS Inc., Rochester, NY).

Results

Minimum inhibitory concentration

The MICs of the CFA derivatives for the five *Staph. 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. CA was the least effective of all compounds tested ($P < 0{\cdot}05$), with MICs of 10 mmol l^{-1} or greater for all bacteria. In contrast, LA had MICs 8–16-fold lower than those of CA.

Table 2 Minimum inhibitory concentration (MIC) values of carbohydrate fatty acid (CFA) derivatives and standards

	Fatty acid		Monoglyceride		CFA derivatives					
Micro-organism	LA	CA	ML	MC	**1**	**2**	**3**	**4**	**5**	**6**
Staphylococcus aureus ATCC 25923	0·63	10	0·04	5	0·31	0·04	0·04	2·5	2·5	0·04
Staph. aureus NCTC 1803	0·63	10	0·04	2·5	0·31	0·04	0·08	1·25	1·25	0·04
Staph. aureus ATCC 33591	1·25	10	0·04	2·5	1·25	0·04	0·04	2·5	5	0·04
Staph. aureus ATCC 33592	1·25	20	0·04	2·5	0·08	0·08	0·04	5	2·5	0·16
Staph. 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 24 h at 35°C.
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.

ML and MC showed greater activity ($P < 0·05$), with values between 0·04–0·08 and 2·5–5 mmol l^{-1} respectively, than their corresponding free fatty acids (LA, CA) with values between 0·63–1·25 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 CFA derivatives with MIC values between 0·04 and 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 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.

Increase in lag time for *Staphylococcus 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 twofold 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 *c.* 2–6 h compared to that of the cultures without treatment. At a fourfold 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 eightfold sub-MIC concentrations (from 1·25 to 0·16 mmol l^{-1}).

Table 3 Increase in lag time ($\Delta\lambda$) for *Staphylococcus aureus* ATCC 25923 in the presence of standards and carbohydrate fatty acid derivatives

Staph. aureus ATCC 25923					
Compound (mmol l^{-1})	$\Delta\lambda$ (h)	SD	Compound (mmol l^{-1})	$\Delta\lambda$ (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	NG		10	NG
ML	0·02	3·9 ± 0·684	MC	1·25	0·0 ± 0·000
	0·04	NG		2·5	1·8 ± 0·986
				5	NG
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	NG
	0·31	NG			
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	NG			

Values are expressed as the difference between the treated culture and culture without compound.
SD, standard deviation; NG, 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.

Leakage of material absorbing at 260 nm

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

Table 4 Effects of carbohydrate fatty acids (CFAs) at 4× minimum inhibitory concentration on membrane integrity in *Staphylococcus 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)		CFA derivatives							
										1		**2**		**3**		**4**	
Staph. aureus	0	0	SD	0	SD	0	SD	0	SD	0	SD	0	SD	0	SD	0	SD
ATCC 25923	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
	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
Staph. aureus	0	0		0		0		0		0		0		0		0	
NCTC 1803	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
	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
Staph. aureus	0	0		0		0		0		0		0		0		0	
ATCC 33591	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
	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
Staph. aureus	0	0		0		0		0		0		0		0		0	
ATCC 33592	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
	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
Staph. aureus	0	0		0		0		0		0		0		0		0	
ATCC 43300	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
	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	ND		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; ND, not determined; SD, standard deviation.

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 *Staph. aureus* suspensions were treated with nisin, LA, ML and CFA derivatives at 4× MIC. The OD_{260} increased rapidly at first, and the absorbance values continued to increase, but more gradually after 60 min. An exception was observed for compound **4**, β-glucose lauric ether, which even after 120 min of exposure did not display a significant increase in $OD_{260\ nm}$.

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 535 nm (G, green) and 615 nm (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 4× MIC for 1 h) showed altered cytoplasmic membrane permeability for LA 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 *c.* 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 260 nm for this compound.

Table 5 Effect of carbohydrate fatty acid derivatives at 4× minimum inhibitory concentration on membrane integrity in *Staphylococcus aureus* ATCC 25923 measured by the BacLight assay

Antimicrobial compounds	Live %	SD
None (nontreated 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.

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 Fig. 2. The alpha lauric derivatives (compounds **1** and **2**) resulted in a noticeable decrease in ATP levels. For the LA 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.

Discussion

The emergence of MRSA, vancomycin-resistant enterococci, 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 CFA esters and

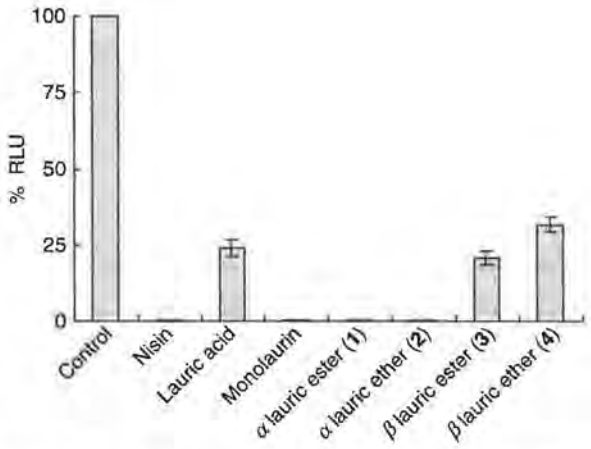

Figure 2 Relative luminescence activity of *Staphylococcus aureus* ATCC25923 cells in response to carbohydrate fatty acid derivatives using the BacTiter-Glo™ assay. Values are expressed as percentage of those obtained with control cultures not exposed to the antimicrobial compounds. **1**, Methyl 6-*O*-lauroyl-α-D-glucopyranoside; **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.

their corresponding ether analogues, against five *Staph. aureus* strains including MRSA strains.

Ruzin and Novick (2000) showed that ML was rapidly hydrolysed ($t_{1/2}$ of *c.* 5 min) by esterases in *Staph. aureus* cultures suggesting that inhibitory activity could be because of free fatty acid liberated from ML 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 (LA and CA).

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 because of 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 ML formulations were active *in vitro* against *Staph. aureus*. Preuss *et al.* (2005) found that Origanum oil, carvacrol, and other essential oils, combined with ML killed *Staph. aureus in vitro*, and proposed that origanum and/or ML, 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 260 nm and the BacLight data, which 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 micro-organism, 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 MRSA to an extent similar to that of the nonresistant strains, suggesting that the mechanisms of methicillin resistance did not affect the CFA derivatives efficacy.

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.

CFA 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.

Acknowledgement

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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

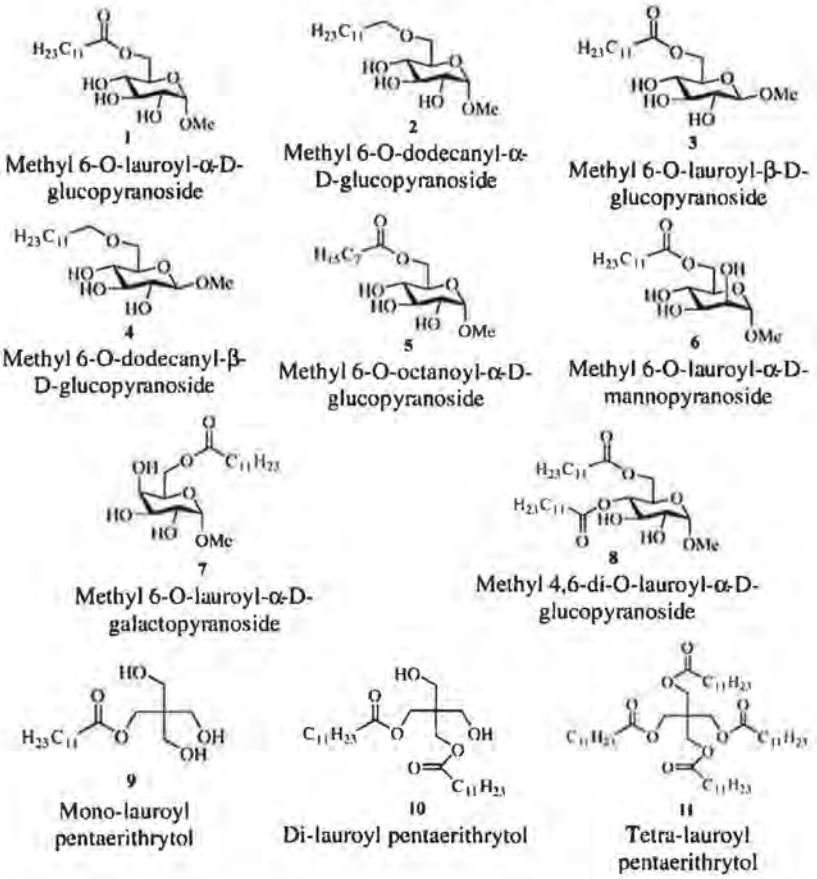

Fig. 1. Structures of the novel carbohydrate fatty acid derivatives and non-carbohydrate polyhydroxylated esters synthesized and investigated.

with test compound to record an increase in OD_{600} of 0.10 *minus* the time that the positive control 1 without test compound required to record the same increase in OD_{600}.

2.5.3. Maximum specific growth rate (μ_{max})

The maximum growth rate was also calculated using the Gen5™ software. The μ_{max} was determined from the slope of the regression equation from the linear portion of the log plot during early exponential phase.

2.5.4. 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. Antimicrobial activity of carbohydrate fatty acid derivatives

3.1.1. Minimum inhibitory concentrations

The MIC results are summarized in Table 2. The monoglycerides, ML and MC, had greater activity ($p<0.05$) against the Gram positive *Listeria* spp. compared to their corresponding free fatty acids (LA, CA), and comparable activity at the concentrations tested against the Gram negative microorganisms. Of the monoglycerides and free fatty acids

Table 2
Minimum Inhibitory Concentration (MIC; mM) values of carbohydrate fatty acid derivatives and standards in tryptic soy broth at 37 °C after 18 h

Microorganism	FA		MG		Carbohydrate fatty acid derivatives					
	LA	CA	ML	MC	1	2	3	4	5	6
Listeria innocua NCTC 11288	0.63	5	0.04	2.5	0.08	0.04	0.08	5	0.63	0.04
Listeria monocytogenes ATCC 7644	0.63	>5	0.04	5	0.08	0.04	0.08	2.5	2.5	0.04
Listeria monocytogenes NCTC 11994	1.25	>5	0.04	2.5	0.31	0.04	0.16	>2.5	1.25	0.04
Listeria monocytogenes NCTC 7973	1.25	5	0.04	2.5	0.08	0.04	0.16	>2.5	0.31	0.04
Escherichia coli ATCC 25922	>20	10	20	5	20	20	20	20	12.5	≥20
Escherichia coli NCTC 12900	12.5	10	12.5	5	12.5	10	12.5	10	12.5	N.D
Salmonella Typhimurium ATCC 14028	>20	>20	20	>20	20	>20	>20	20	>20	N.D
Enterobacter aerogenes ATCC 13048	>20	20	20	10	20	>20	>20	>20	>20	N.D
Pseudomonas fluorescens	>20	5	20	5	>20	>20	>20	>20	5	N.D

For each analysis the MIC was recorded as the concentration (mM) that resulted in total inhibition of all replicates. N.D: Not determined.
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.

tested, ML had the lowest MIC values ($p<0.05$) and was particularly effective for inhibition of *Listeria* strains with MIC values of 0.04 mM, by comparison with the range observed for LA with MIC values between 0.63 mM to 1.25 mM. A similar trend was observed for MC (MIC = 2.5 mM, 5.0 mM) compared to the free fatty acid CA (MIC ≥ 5 mM).

When tested against the Gram negative bacteria, LA and ML had no activity at concentrations up to 20 mM (Table 2). An exception to this was recorded for *E. coli* NCTC12900 with a MIC value of 12.5 mM for LA and ML. *P. fluorescens* was susceptible to CA and MC at a concentration of 5 mM for both compounds, whereas for *E. coli* strains, MIC values were 10 mM and 5 mM respectively. Minimum inhibitory concentrations of CA were ≥20 mM for the other Gram negative bacteria (Table 2).

All CFA derivatives showed greater antimicrobial activity against Gram positive microorganisms than Gram negative ($p<0.05$). For *Listeria* spp., compounds **2** and **6** were the most active derivatives with MIC values of 0.04 mM, comparable to ML (Table 2). The next in order of overall efficacy was compound **3** with MIC values between 0.08 mM and 0.16 mM for *Listeria* spp. Compound **1** recorded an MIC range of 0.08 mM to 0.31 mM. The antimicrobial activity of compound **4** was significantly lower than that observed with the corresponding α-ether (Table 2). Compound **9** (a non-carbohydrate mono-ester) was evaluated, but its antimicrobial activity was negligible (results not shown). Compounds **7**, **8**, **10** and **11** could not be accurately tested for antimicrobial efficacy due to poor solubility in water. Compound **5** had a greater activity ($p<0.05$) compared with MC against all *Listeria* strains (Table 2). Compound **5** was more active than the lauric acid derivatives against *E. coli* ATCC 25922 and *P. fluorescens*, with MIC values of 12.5 mM and 5 mM respectively (Table 2).

Table 3
Effect of FA, MG and CFA derivatives on the Lag time (λ) and Maximum specific growth rate (μ_{max}) of *L. monocytogenes* ATCC 7644

Compound	(mM)	λ (h)	St. Dev.	μ_{max} (h^{-1})	St. Dev.
LA	0	–		0.30	±0.034
	0.04	0.0	±0.06	0.22	±0.049
	0.08	0.2	±0.26	0.17	±0.041
	0.16	2.0	±1.00	0.10	±0.017
	0.31	4.8	±1.73	0.07	±0.037
	0.63	No growth		0	
ML	0	–		0.30	±0.034
	0.02	2.3	±1.09	0.25	±0.040
	0.04	No growth		0	
1	0.08	No growth		0	
2	0.04	No growth		0	
3	0	–		0.30	±0.034
	0.02	0.5	±0.07	0.31	±0.003
	0.04	5.3	±0.67	0.27	±0.006
	0.08	No growth		0	
4	0	–		0.30	±0.034
	0.16	0.2	±0.18	0.31	±0.013
	0.31	0.5	±0.25	0.27	±0.009
	0.63	5.0	±0.55	0.12	±0.059
	1.25	No growth		0	
CA	0	–		0.30	±0.034
	0.31	0		0.26	±0.027
	0.63	0	±0.04	0.24	±0.037
	1.25	0.1	±0.17	0.26	±0.044
	2.5	0.8	±0.19	0.21	±0.034
	5	3.1	±1.62	0.18	±0.097
	10	No growth		0	
MC	0	–		0.30	±0.034
	0.31	0.2	±0.29	0.26	±0.029
	0.63	0.3	±0.40	0.25	±0.043
	1.25	1.1	±0.41	0.19	±0.046
	2.5	5.6	±1.35	0.01	±0.034
	5	No growth		0	
5	0	–		0.30	±0.034
	0.31	0.4	±0.47	0.24	±0.035
	0.63	1.6	±0.94	0.22	±0.008
	1.25	1.0	±0.27	0.12	±0.008
	2.5	1.9	±0.34	0.08	±0.001
	5	No growth		0	

In each antimicrobial efficacy assay, the corresponding carbohydrates for the fatty acid derivatives were included as a control, but had no antimicrobial or growth promoting effect on the microorganisms under investigation. Although the concentrations of ethanol corresponding to that within the wells with the highest concentrations of compound used (10 mM for the Gram positive and 20 mM for the Gram negative bacteria) had a minor effect on bacteria viability, there was no anti-microbial effect observed at the concentrations used when incorporated with the compounds at MIC levels.

3.1.2. Increase in Lag time and decrease of maximum specific growth rate

The increase in lag time and decrease in maximum specific growth rate was estimated for *L. monocytogenes* ATCC 7644 to allow further comparison between compound efficacies. Results were found to be concentration and compound dependent (Table 3) ($p<0.05$). Generally, the increase in lag time between concentrations of a compound was observed to be more marked than the decrease in growth rate which was more gradual. For example, at sub-MIC concentrations, compound **3** had an increase in lag time from 0.5 h to 5.3 h associated with a small increase in concentration from 0.02 mM to 0.04 mM. This trend was also true for LA, CA, MC and compound **4** (Table 3). With respect to μ-max, different patterns were observed, there was a gradual decrease noted with LA, CA, MC and compound **4**, associated with the higher MIC values for these compounds. Whereas, for ML and compound **3**, there was a non-linear association of μ-max reduction with concentration, associated with the very low MIC values determined for these compounds.

4. Discussion

The antimicrobial potential of carbohydrate fatty acid derivatives has received less attention than their other functional properties as emulsifiers or non-ionic surfactants. In contrast to the extensive literature for the antimicrobial properties of monoglycerides, there is limited information about the use of CFA derivatives as food preservatives. Previous studies on antimicrobial properties of sugar esters mainly involved sucrose or other disaccharides esters (Hathcox and Beuchat, 1996; Devulapalle et al., 2004). Many of the studies were not carried out using regiochemically pure compounds, were not quantitative and did not include controls to compare activity of free fatty acids with fatty acid derivatives. As a result correlation of chemical structure with efficacy and/or mechanism of action has been difficult.

The current study evaluated the antimicrobial properties of pure fatty acid esters and their corresponding ethers to provide insights into structure/activity relationships for these compounds. The CFA derivatives synthesized in this study were shown to be more effective against Gram positive than Gram negative bacteria ($p<0.05$). This trend was also observed for the fatty acid and monoglyceride controls, in accordance with previous studies (Conley and Kabara, 1973; Ruzicka et al., 2003). We obtained similar MIC values of 10 μg/ml for monolaurin against *L. monocytogenes* as those reported by Wang and Johnson (1992), and Oh and Marshall (1993). The activity of lauric derivatives **2** and **6** against *L. monocytogenes* was found to be equivalent to that of monolaurin and in excess of that reported by Monk et al. (1996), for a lauroyl-sucrose ester.

With respect to the effect of chain length on antimicrobial efficacy of the CFA's, there was a difference in efficacy between Gram positive and Gram negative bacteria. Lauric acid and derivatives had higher activity against Gram positive bacteria, whereas caprylic acid and its derivative **5** were more active than lauric acid derivatives against *E. coli* ATCC 25922 and *P. fluorescens*. Our data are similar to that of Nair et al. (2004a), where populations of *L. monocytogenes* and *E. coli* O157:H7 were shown to decrease below detection levels using 50 mM of MC or CA in bovine milk. The same authors, Nair et al. (2005), described antimicrobial activity for both CA and MC and found that *Streptococcus* spp. were the most sensitive, and *E. coli* the most tolerant. Whilst both lauric and caprylic fatty acid derivatives retained good activity against Gram positive bacteria, only the caprylic acid derivative displayed useful

efficacy against Gram negative bacteria. These trends were also observed with the free FAs and MGs. The enhanced efficacy of the shorter chain fatty acid over the medium chain fatty acid could be attributed to the differences in the outer membrane structure and permeability between Gram-negative and Gram-positive bacteria.

This study also looked at fatty acids conjugated to sugars by ether bonds. Such bonds are not as readily hydrolyzed in biological systems as their ester equivalents. It was interesting to note that these compounds still retained antimicrobial activity indicating that hydrolysis of the ester bond is not necessary for antimicrobial activity. Compound **4** (β ether) was less inhibitory than the free fatty acid (LA) and monoglyceride (ML) against *Listeria* spp. In some cases, compound **2** (α ether) had an enhanced activity by comparison with compound **1** (α ester) and **3** (β ester), particularly for the *Listeria* spp. This may be due to the greater stability of ether bonds over esters (Ved et al., 1984), since ether bonds are not subject to cleavage by cellular esterases. Reporting on the antimicrobial efficacy of ether and ester glyceride compounds, Isaacs et al. (1995), suggested that ether lipids should remain antimicrobial for a longer period of time than monoglycerides with ester linkages, which assumes that the fatty acid component does not require release, for example, by esterases for activity. Ruzin and Novick (2000) showed that monolaurin was rapidly hydrolyzed ($t_{1/2}$ of ~5 min) by esterases in *S. aureus* suggesting that inhibitory activity could be due to free fatty acid liberated from monolaurin by hydrolysis. The differences observed in this study between the ester and ether bonds of the same carbohydrate fatty acid (compounds **1** and **2** and compounds **3** and **4**) show that the nature of the bond between the fatty acid and the sugar has an influence on antimicrobial activity.

The focus of many studies on the mechanism of action of monoglycerides is on cellular membranes. Ruzin and Novick (2000) reported a monolaurin esterase activity in association with the cell membrane and also in the cytoplasm and the Geh lipase was responsible for approximately 80% of the monolaurin hydrolysing activity. The same authors reported increased lipolytic activity in membrane fractions of *S. aureus* and concluded that *S. aureus* had a membrane bound esterase that participated in the hydrolysis of monolaurin and release of lauric acid. However, the current work suggests that while membrane bound or free esterases may cleave ester bonds of a glycerol or a carbohydrate fatty acid derivative, the ether carbohydrate fatty acid derivatives retained higher activity than the ester derivatives and that the release of a free fatty acid may not be required for potent antimicrobial activity.

In an effort to probe the importance of the carbohydrate moiety, ester and ether fatty acid derivatives based on the following carbohydrates were synthesized and tested: α-glucose, β-glucose, α-mannose and α-galactose. Of these, differences in efficacy were measured for compounds which have the same glycoconjugate bond and alkyl chain length (see entries in Table 2 for compounds **1**, **3**, **6**, **7**). Therefore we conclude that the sugar itself can be a determining factor on efficacy. This is in accordance with the findings of Watanabe et al. (2000) who also concluded that the configuration of the carbohydrate moiety in similar compounds markedly affected antibacterial activity. In addition, we found that a minor structural change in the carbohydrate can have a major influence on the solubility of the compound. For example, compounds **1**, **3**, and **6** are soluble, whereas the structurally similar compound **7** is insoluble. This further highlights the importance of the choice of carbohydrate.

We found that not only were free single or multiple hydrophilic groups necessary for biological activity, as observed by Conley and Kabara (1973), but that the nature of the hydrophilic group *per se* is also important for the antibacterial activity, as antimicrobial activity associated with the lauroyl pentaerythritol monoester **9** with three free hydroxyl groups was negligible compared to compounds **1**, **3** and **6** which also had the same number of free hydroxyl groups.

Results for compound **8** demonstrates that there is a limit to the number of fatty acids which can be esterified to a monosaccharide and this appears to be one, whereas for the sucrose it has been demonstrated that it is two (Kato and Shibasaki, 1975). Due to the poor solubility in water of compounds **7**, **8**, **10** and **11**, their potential for application in food systems is limited.

The data obtained from the increase in λ and decrease in μ-max studies showed that sub-MIC concentrations can modify bacterial growth significantly. Nair et al. (2004b) also observed this behaviour using MC (50 mM) which reduced *Enterobacter sakazakii* in reconstituted infant formula by >5 log CFU/ml at 37 °C, whereas approximately 1.5 log CFU/ml of the pathogen survived after 24 h of incubation using half the concentration of antimicrobial. This is important towards possible combinations with other antimicrobials or alternative preservation strategies for optimization of practical application of CFA derivatives to microbiological issues within the food and other industries. Combinations of sub-MIC preservatives with other minimal 'hurdles' may contribute to the control of microbiological issues in food systems while minimizing sensory and quality impacts on a food. Combinations of LA or a derivative and other antimicrobials have shown additive or synergistic effects against pathogenic or spoilage bacteria in several matrices (Bell and De Lacy, 1987, Wang and Johnson, 1997; Blaszyk and Holley, 1998; Yamazaki et al., 2004).

Lauric esters of methyl glucopyranoside (**1** and **3**) had comparable activity ($p>0.05$) against all Gram positive bacteria tested, regardless of the anomeric configuration of the sugar. With regard to the lauric ethers, compound **2** showed lower MIC values (0.04 mM) against the Gram positive microorganisms compared to compound **4** (2.5 mM to 5 mM, $p<0.05$). This suggests that the alpha or beta configuration of the ether derivative has a considerable effect on the anti-microbial efficacy. In general, the alpha configuration of the carbohydrate moiety of the synthesized compounds was more effective than the beta, for both ester and ether derivatives of the same carbohydrate. This further supports the observation that the carbohydrate moiety has a role in the antimicrobial efficacy of the carbohydrate fatty acid derivative. This finding suggests that there is potential to develop carbohydrate fatty acid derivatives with an efficacy comparable to that of glycerol fatty acid derivatives such as monolaurin.

5. Conclusions

A series of pure, regiochemically defined monosaccharide monofatty acid esters and their corresponding ethers were evaluated for antimicrobial activity. The CFA derivatives were found to be significantly more active against Gram positive bacteria than Gram negative bacteria, and lauric esters of methyl glucopyranoside and mannopyranoside as well as the lauric ether of methyl glucopyranoside were comparable to Monolaurin for antimicrobial efficacy. 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. No significant variability in the efficacy of the compounds was observed between *Listeria* strains. The use of a synthetic route to control production of regiochemically defined compounds allows the optimization of the carbohydrate moiety configuration and bond with regard to anti-microbial efficacy, highlighting compounds suitable for regioselective enzymatic synthesis. Carbohydrate fatty acid derivatives have potential as effective antimicrobial compounds for use as preservatives to address a range of microbiological stability and safety issues. Additional knowledge on the mode of action of such compounds in combination with data on their MICs would allow for effective applications.

Acknowledgement

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Synthesis and antimicrobial evaluation of carbohydrate and polyhydroxylated non-carbohydrate fatty acid ester and ether derivatives

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ABSTRACT

A series of fatty acid ester and ether derivatives have been chemically synthesised based on carbohydrate and non-carbohydrate polyhydroxylated scaffolds. The synthesised compounds, along with their corresponding fatty acid monoglyceride antimicrobials, were evaluated for antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. Of the derivatives synthesised, several of the carbohydrate-based compounds have antimicrobial efficacy comparable with commercially available antimicrobials. The results suggest that the nature of the carbohydrate core plays a role in the efficacy of carbohydrate fatty acid derivatives as antimicrobials.

1. Introduction

The antimicrobial effects of fatty acids have been well documented.[1] Generally, long-chain fatty acids have activity against Gram-positive bacteria, while short-chain fatty acids are more active against Gram-negative bacteria. Lauric acid (a medium-chain fatty acid) is regarded as the most active, with reported activity against both Gram-positive and Gram-negative bacteria.[2] Lauric acid and gentamicin combined have been reported to show activity against MRSA.[3] Lauric acid is inexpensive and, therefore, may be very useful for infection control in hospitals.

Esterification of fatty acids with monohydric alcohols such as methanol or ethanol has been shown to reduce their antimicrobial activity.[4] In contrast, esterification of fatty acids to the polyhydric alcohol glycerol increased their effectiveness.[5] One of the most active of these antimicrobial derivatives is monolaurin (Lauricidin®), the glycerol monoester of lauric acid, which is used as a key ingredient of antimicrobial food additives to inhibit the growth of undesirable microorganisms.[6,7]

More recently, a study has shown that the corresponding ether of monolaurin, dodecylglycerol, had greater potency against *Streptococcus faecium* than monolaurin itself, albeit depending on the incubation conditions.[8] The greater potency of dodecylglycerol was ascribed to its greater retention by the cell and its action on specific receptors or enzymes.

Another class of fatty acid derivatives that has broad applications in the food industry is that of the carbohydrate fatty acid esters.[9,10] While they are most commonly employed as surfactants, their antimicrobial properties have been documented.[11] The use of carbohydrate esters is increasingly favoured since they are biodegradable, are not harmful to the environment and are non-toxic.[12]

The most common carbohydrate fatty acid esters utilised to date are sucrose esters. They are commercially available and used for a variety of food applications. Kato and Shibasaki showed that the sucrose ester of lauric acid had potent antimicrobial activity against certain Gram-positive bacteria and fungi. They further showed that, in contrast to findings with glycerides, the diester of sucrose was more active than the monoester. Of the diesters tested, sucrose dicaprylate showed the highest activity.[13]

Other oligosaccharide fatty acid esters, including maltose and maltotriose, have been synthesised. These sugar esters were shown to inhibit the growth of *Streptococcus sobrinus* and are therefore potentially of significant value in the development of oral-hygiene products.[14] One study investigating the effect of carbohydrate monoesters reported that among those synthesised, galactose laurate, fructose laurate and the reducing 6-*O*-lauroylmannose showed the highest inhibitory effect against *Streptococcus mutans*, while other analogues of hexose laurates showed no activity.[15] This finding strongly suggests that the carbohydrate moiety can markedly affect the antimicrobial activity of the fatty acid, and, therefore, further investigation is merited.

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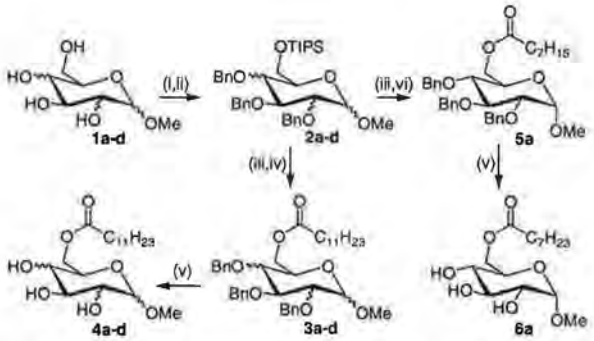
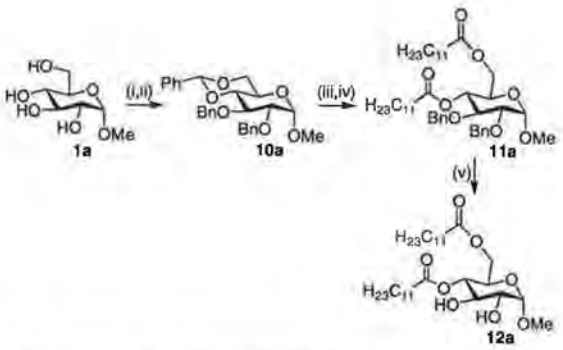


Scheme 1. Reagents and conditions: (i) DMF anhyd, TIPSCl, imidazole, rt; (ii) anhyd DMF, NaH, BnBr, rt; (iii) THF anhyd, 0 °C, TBAF, rt; (iv) anhyd Pyr, DMAP, Lauroyl Cl, rt; (v) EtOH, Pd/C, H_2; (vi) Pyr anhyd, DMAP, octanoyl Cl, rt.



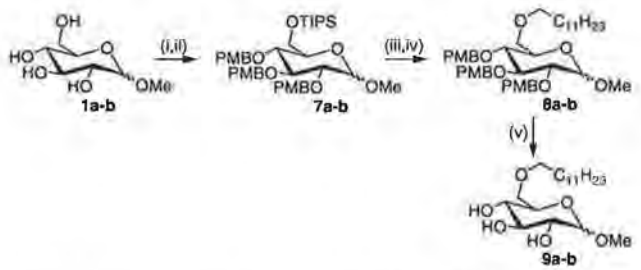
Scheme 3. Reagents and conditions: (i) pTSA, $PhCH(OMe)_2$, anhyd MeCN, rt; (ii) anhyd DMF, NaH, BnBr, rt (95% yield over 2 steps); (iii) MeOH, TsOH; (iv) anhyd Pyr, DMAP, lauroyl Cl, rt (38% yield over 2 steps); (v) EtOH, Pd/C, H_2 (75% yield).

Recent work in the area of carbohydrate fatty acid esters has focused on establishing an effective regioselective, enzyme-catalysed synthesis of sugar derivatives for use as surfactants for industrial applications;[16–20] however, relatively few studies have examined role of the carbohydrate in antimicrobial activity.[14,21,22]

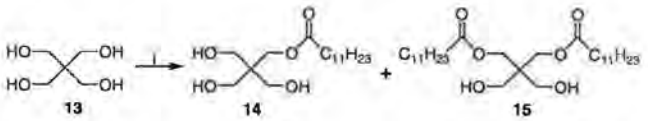
This study is concerned with the synthesis of carbohydrate and polyhydroxylated non-carbohydrate fatty acid derivatives for evaluation as antibacterial agents, with a view to examining the effect of variation of the hydrophilic moiety on antimicrobial activity. Therefore, we designed chemical syntheses to investigate the effects of carbohydrate versus non-carbohydrate hydrophilic cores, the number of fatty acids attached to the hydrophilic core, the monosaccharide core itself (and the anomeric configuration with respect to glucopyranoside), the glycoconjugate linkage and the length of fatty acid chain on antimicrobial activity.

A quantitative assay for antimicrobial activity was used to allow comparisons between compounds, and all were measured relative to the free fatty acids and monolaurin as reference compounds.

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. For this study, we have developed a chemical route to allow the synthesis of a number of pure, regio-defined, monosaccharide mono fatty acid esters (Scheme 1). We have also developed a route to the corresponding ether derivatives (Scheme 2). In order to establish whether a second fatty acid conjugated to a monosaccharide would improve antimicrobial activity, a route was developed to synthesise a di-laurate derivative (Scheme 3). Furthermore, to investigate whether the structure and, therefore, the synthesis could be simplified and retain activity, non-carbohydrate hydroxylated esters based on a pentaerythritol core were synthesised by a straightforward esterification (Scheme 4).



Scheme 2. Reagents and conditions: (i) DMF anhyd, TIPSCl, imidazole, rt; (ii) anhyd DMF, THF anhyd, 0 °C, NaH, PMBCl, TBAI, rt; (iii) anhyd THF, 0 °C, TBAF, rt; (iv) anhyd DMF, dodecanyl chloride, 0 °C, NaH, rt; (v) 3:1 MeCN–H_2O, CAN, rt.



Scheme 4. Reagents and conditions: (i) Pyr anhyd, DMAP, lauroyl Cl, rt (**14**, 14%; **15**, 29%).

2. Results and discussion

2.1. Synthesis

A chemical route designed to obtain monoester sugars is shown in Scheme 1 and is based on the following carbohydrate starting materials: methyl α-D-glucopyranoside (**1a**), methyl β-D-glucopyranoside (**1b**), methyl α-D-mannopyranoside (**1c**) and methyl α-D-galactopyranoside (**1d**). The synthesis commenced with the selective protection of the primary hydroxyl group of sugars **1a–d** with a triisopropylsilyl (TIPS) group. The silyl derivatives were then fully protected with benzyl groups to give **2a–d**. The removal of the TIPS group by tetrabutylammonium fluoride in THF allowed for the esterification of the free 6-OH position with either lauroyl chloride to yield **3a–d** or octanoyl chloride to yield **5a**. Removal of the benzyl groups by catalytic hydrogenation led to the unprotected carbohydrate esters **4a–d** and **6a**, respectively (Table 1).

Synthesis of the ether derivatives also commenced with the protection of the primary hydroxyl group with a triisopropylsilyl group (Scheme 2). The sugars were then fully protected using *p*-methoxybenzyl chloride (PMB), to yield **7a–b**. Removal of the TIPS group gave the free primary hydroxyl. Next, the lauryl ether group was attached using dodecanyl chloride to give the fully protected ether derivatives **8a–b**. Finally, oxidative cleavage of the PMB groups with ceric ammonium nitrate (CAN) gave the monododecanyl sugars **9a–b** (Table 2).

The method used to synthesise di-*O*-lauroyl derivative **12a** is shown in Scheme 3. The 4 and 6-OH positions of methyl α-D-glucopyranoside (**1a**) were protected with a benzylidene group using benzaldehyde dimethylacetal. The remaining free OHs were then converted to benzyl ethers to give **10a**. Removal of the benzylidene acetal using a catalytic amount of TsOH in MeOH then enabled the esterification of the 4- and 6-OH to give **11a**. Finally, removal of the benzyl groups by catalytic hydrogenation gave the diester derivative **12a**.

Direct esterification of pentaerythritol **13**, using lauroyl chloride and DMAP in pyridine, yielded the non-sugar derivatives **14** and **15** as shown in Scheme 4.

Table 1
Percentage yields of compounds **2a–d**, **3a–d**, **4a–d**, **5a** and **6a**

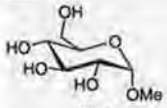
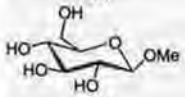
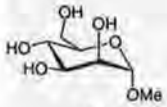
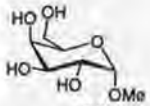
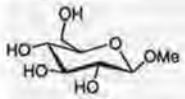
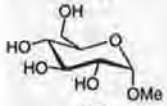
Carbohydrate (1)	2,3,4-Tri-*O*-Bn-6-*O*-TIPS (2)	2,3,4-Tri-*O*-Bn-6-*O*-lauroyl (3)	6-*O*-Lauroyl (4)	2,3,4-Tri-*O*-Bn-6-*O*-octanoyl (5)	6-*O*-Octanoyl (6)
 **(1a)**	**2a** 85%	**3a** 72%	**4a** 86%	**5a** 63%	**6a** 73%
 **(1b)**	**2b** 80%	**3b** 70%	**4b** 75%		
 **(1c)**	**2c** 51%	**3c** 64%	**4c** 75%		
 **(1d)**	**2d** 50%	**3d** 60%	**4d** 86%		

Table 2
Percentage yields of compounds **7a–b**, **8a–b** and **9a–b**

Carbohydrate (1)	2,3,4-Tri-*O*-PMB-6-*O*-TIPS (7)	2,3,4-Tri-*O*-PMB-6-*O*-dodecanyl (8)	6-*O*-Dodecanyl (9)
 **(1b)**	**7a** 59%	**8a** 50%	**9a** 73%
 **(1a)**	**7b** 61%	**8b** 85%	**9b** 76%

2.2. Antimicrobial activity of the fatty acid derivatives

Two non-carbohydrate polyhydroxylated fatty acid ester derivatives, six carbohydrate fatty acid ester derivatives and two carbohydrate long-chain alkyl ether derivatives, together with their corresponding polyhydric alcohols, fatty acids and monoglycerides as controls, were tested against a Gram-positive bacterium, *Staphylococcus aureus*, and a Gram-negative bacterium, *Escherichia coli*, to assess their antimicrobial activity. The efficacy of the derivatives and controls was compared using minimum inhibitory concentration values (MIC), which is defined as the lowest concentration of compound that shows no increase in cell growth for all the replicates compared to a negative control after 18 h.

The polyhydric alcohols (carbohydrates and pentaerythritol) showed no antimicrobial activity or growth-promoting effects for the microorganisms under the conditions used (results not shown).

The data in **Table 3** show that the monoglycerides, monolaurin and monocaprylin, had greater activity compared to the free fatty acids, lauric acid and caprylic acid, against *S. aureus*. Of the monoglycerides and free fatty acids tested, monolaurin had the lowest MIC values for *S. aureus*, with a value of 0.04 mM compared to a value of 0.63 mM for lauric acid. Furthermore, monocaprylin showed MIC values of 2.5 mM against *S. aureus* compared to the value of 5.0 mM for caprylic acid. With respect to *E. coli*, monolaurin showed less inhibitory effect than lauric acid with values of 20 mM and 10 mM, respectively. In contrast, monocaprylin showed activity against *E. coli* at concentrations of 6.25 mM compared with caprylic acid value of 12.5 mM.

Table 3
MIC values of fatty acid derivatives and controls

Compound	*S. aureus* ATCC 25923 (mM)	*E. coli* ATCC 25922 (mM)
Lauric acid	0.63	10
Monolaurin	0.04	20
Caprylic acid	5	12.5
Monocaprylin	2.5	6.25
Methyl 6-*O*-lauroyl-α-D-glucopyranoside (**4a**)	0.31	20
Methyl 6-*O*-lauroyl-β-D-glucopyranoside (**4b**)	0.04	20
Methyl 6-*O*-octanoyl-α-D-glucopyranoside (**6a**)	2.5	12.5
Methyl 6-*O*-dodecanyl-α-D-glucopyranoside (**9a**)	0.04	20
Methyl 6-*O*-dodecanyl-β-D-glucopyranoside (**9b**)	2.5	20
Methyl 4,6-di-*O*-lauroyl-α-D-glucopyranoside (**12a**)	ND[a]	ND
Methyl 6-*O*-lauroyl-α-D-mannopyranoside (**4c**)	0.04	20
Methyl 6-*O*-lauroyl-α-D-galactopyranoside (**4d**)	>10	>20
Monolauroyl pentaerythritol (**14**)	>10	>20
Dilauroyl pentaerythritol (**15**)	ND	ND

[a] Not determined due to insolubility.

All fatty acid derivatives showed greater antimicrobial activity against *S. aureus* than *E. coli*.

Among the sugar fatty acid esters and the sugar alkyl ethers prepared, methyl 6-*O*-dodecanyl-α-D-glucopyranoside (**9a**), methyl 6-*O*-lauroyl-α-D-mannopyranoside (**4c**) and methyl 6-*O*-lauroyl-β-D-glucopyranoside (**4b**) showed the best inhibitory effects for *S. aureus*, with MIC values of 0.04 mM. The next derivative in order of efficacy was methyl 6-*O*-lauroyl-α-D-glucopyranoside (**4a**), with a value of 0.31 mM. Methyl 6-*O*-octanoyl-α-D-glucopyranoside 6a was comparable to monocaprylin against *S.*

aureus with values of 2.5 mM. This compound was also more active than any of the lauric acid derivatives against *E. coli*. Methyl 6-*O*-dodecanyl-β-D-glucopyranoside (**9b**) gave similar results as those for **6a** for *S. aureus* with values of 2.5 mM. The galactopyranoside ester derivative **4d** and the mono-*O*-lauroyl pentaerythritol **14** were the least active compounds tested, both with comparatively negligible MIC values of >10 mM for *S. aureus* and >20 mM for *E. coli*.

The di-substituted methyl 4,6-di-*O*-lauroyl-α-D-glucopyranoside (**12a**) did not show any activity comparable with either the monoglycerides or indeed the monosubstituted sugar derivatives. This was attributed to poor solubility in water, as was the case for the di-substituted non-sugar compound di-lauroyl pentaerythritol **15**.

2.3. Discussion

In this present study, we have evaluated the effect of polyhydroxylated fatty acid derivatives as inhibitors of a Gram-positive (*S. aureus*) and a Gram-negative (*E. coli*) microorganism of concern to the food and healthcare industries. Several of the synthesised compounds have antimicrobial efficacies comparable with commercially available antimicrobials against *S. aureus*.

We studied the effect of carbohydrate versus non-carbohydrate hydrophilic cores (carbohydrate and pentaerythritol laurates), the degree of substitution (monoester and diester), the monosaccharide core (glucopyranoside, mannopyranoside and galactopyranoside), the anomeric configuration (α and β glucopyranoside), the type of fatty acid carbohydrate linkage (ester and ether) and the length of fatty acid chain (lauric and caprylic) on antimicrobial activity.

As with the monoglycerides and free fatty acids, all of the fatty acid derivatives that were found to be active showed greater antimicrobial activity against *S. aureus* than *E. coli*.

The non-carbohydrate pentaerythritol monoester **14**, which has the same number of free hydroxyl groups as the carbohydrate monoester derivatives, showed negligible activity against both microorganisms tested, indicating that the carbohydrate itself could play an important role in the antimicrobial activity of these compounds.

The degree of substitution of these derivatives was also shown to be crucial as both the non-sugar pentaerythritol diester **15** and the carbohydrate methyl α-D-glucopyranoside diester (**12a**) were much less soluble in water than the monoesters. As a consequence, no antimicrobial activity results for these compounds could be obtained.

With regard to the influence of different sugar cores, the results showed that the lauric ester derivative of methyl α-D-mannopyranoside (**4c**) and methyl β-D-glucopyranoside (**4b**) showed higher activity than any other ester derivatives against *S. aureus*, supporting the observation that the nature of the carbohydrate is involved in the antimicrobial efficacy of the derivatives. This conclusion is consistent with results of an earlier study by Watanabe et al.[15]

Further evidence for this is noted in the results for the lauric ester anomers of methyl glucopyranosides **4a** and **4b**. A difference was noted when these compounds were tested against *S. aureus*, with the beta configuration showing higher activity. The lauric ether anomers of methyl glucopyranosides **9a** and **9b** also showed a marked difference in activity when tested against *S. aureus*, with the alpha configuration showing a much higher activity.

In addition, the difference in activity between the ester and ether conjugates of the same carbohydrate showed that for the methyl α-D-glucopyranoside derivatives, the ether derivative **9a** was more active than the ester **4a**; however, for methyl β-D-glucopyranoside, the ester 4b was more active than the ether **9b**. These results indicate that, in combination with other factors, the nature of the bond conjugating the fatty acid to the carbohydrate could play some role in antimicrobial activity.

The importance of the chain length of the fatty acid ester was investigated using both lauric and caprylic derivatives. The lauric ester derivative **4a** showed much higher activity against *S. aureus* compared to the corresponding caprylic ester derivative **6a**. Conversely, the caprylic ester derivative **6a** showed higher activity against *E. coli* compared with the lauric derivative **4a**. This trend was also observed for the monoglyceride controls and is in accordance with general trends observed for medium- and short-chain fatty acids.[2]

In conclusion, these results suggest that the nature of the carbohydrate core plays a role in the efficacy of carbohydrate fatty acid derivatives such as antimicrobials, and therefore further optimisation may be possible. However, to confirm the trends outlined with respect to the importance of the carbohydrate moiety and the role of the nature of the glycoconjugate bond, further studies are warranted using a wider range of Gram-positive and Gram-negative microorganisms, which would allow for evaluation of potential species and strain effects.

3. Experimental

3.1. Synthesis

3.1.1. General methods

All air- and moisture-sensitive reactions were performed under an inert nitrogen atmosphere. All reactions performed under a hydrogen atmosphere were performed in a Parr hydrogenator. Anhydrous DMF, THF, pyridine and MeCN were purchased from Sigma–Aldrich. TLC was performed on aluminium sheets precoated with Silica Gel 60 (HF_{254}, Fluka), and spots were visualised by UV and charring with 1:20 H_2SO_4–EtOH. Flash column chromatography was carried out with Silica Gel 60 (0.040–0.630 mm, E. Merck) using a stepwise solvent polarity gradient correlated with TLC mobility. Chromatography solvents used were EtOAc (Riedel-de-Haen), MeOH (Riedel-deHaen) and petroleum ether (bp 40–60 °C, Fluka). Optical rotations were determined with an AA-% Series Optical Activity Ltd polarimeter. NMR spectra were recorded with Varian Inova 300 and Varian AS 400 spectrometers. Chemical shifts are reported relative to internal Me_4Si in $CDCl_3$ (δ 0.0) for ^1H and $CDCl_3$ (δ 77.0) for ^{13}C. Coupling constants are reported in hertz (Hz). FTIR spectra were recorded with a Nicolet FT-IR 5DXB infrared spectrometer, samples were prepared in a KBr matrix. Low-resolution electrospray-ionisation mass spectra (LRESIMS) were measured in the positive-ion mode on a Micromass Quattro tandem quadropole mass spectrometer. Methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, methyl α-D-mannopyranoside, methyl α-D-galactopyranoside, pentaerythritol, 1-chlorododecane, lauroyl chloride and octanoyl chloride were purchased from Sigma–Aldrich.

3.1.2. Methyl 2,3,4-tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-glucopyranoside (2a)

A solution of **1a** (5 g, 25 mmol) in anhyd DMF (120 mL) was treated with chlorotriisopropylsilane (TIPSCl, 15 mL, 75 mmol) and imidazole (5 g, 75 mmol) and allowed to stir at room temperature for 24 h. The crude TIPS-protected intermediate was then concentrated in vacuo and dissolved in EtOAc. The organic extract was washed with 10% HCl and water, followed by satd aq $NaHCO_3$ and finally satd aq NaCl. It was then dried over anhyd $MgSO_4$ and concentrated under reduced pressure.[23] The crude product was dissolved in anhyd DMF (50 mL) and cooled to 0 °C. NaH (5 g,

125 mmol) was added portionwise, BnBr (9 mL, 75 mmol) was added, and the mixture was allowed to warm to room temperature and stirred for 24 h. MeOH (50 mL) was added to quench the reaction, and the mixture was stirred for 1 h. The solution of the fully protected sugar was then concentrated in vacuo, and the residue was dissolved in EtOAc. The solution was washed with water, dried over anhyd $MgSO_4$ and concentrated under diminished pressure.[24] The resulting crude product was purified by chromatography (petroleum ether–EtOAc) to give **2a** (13.2 g, 85%); $[\alpha]_D$ 10.7 (*c* 0.07, $CHCl_3$); FTIR (KBr): 2923, 1733, 1498, 1455, 909, 884, 791, 695 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.36–7.27 (ms, 15H, aromatic H), 4.91, (AB d, 2H, *J* 11.0, OCH_2Ph), 4.78, (AB d, 2H, *J* 11.0, OCH_2Ph), 4.74 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.61 (d, 1H, $J_{1,2}$ 3.5, H-1), 3.99 (apt t, 1H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.5, H-3), 3.84 (d, 2H, $J_{5,6}$ 4.5, H-6a,6b), 3.64 (m, 1H, H-5), 3.55–3.49 (overlapping signals, 2H, H-2,4), 3.37 (s, 3H, OCH_3), 1.10–1.02 (ms, 18H, each TIPS CH_3), 0.88 (m, 3H, each TIPS *CH*); ^{13}C NMR ($CDCl_3$): δ 139.1, 138.7, 138.5 (each s, each aromatic C), 128.65, 128.63, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8 (each d, each aromatic CH), 98.0 (d, C-1), 82.5, 80.5, 78.1, 76.1 (each d), 76.1, 75.3, 73.6 (each t, each CH_2Ph), 62.9 (t, C-6), 55.0 (q, OCH_3), 18.3, 18.2 (each q, each TIPS CH_3), 12.2 (each d, each TIPS CH); LRESIMS: Found, *m/z* 643.3; required, 643.9 $[M+Na]^+$.

3.1.3. Methyl 2,3,4-tri-*O*-benzyl-6-*O*-triisopropylsilyl-β-D-glucopyranoside (2b)

Treatment of **1b** (4.5 g, 23.17 mmol) as described for **1a** gave **2b** (8.7 g, 80%); $[\alpha]_D$ 23 (*c* 0.01, $CHCl_3$); FTIR (KBr): 2863, 1730, 1497, 1454, 1399, 1277, 882, 802, 751, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.37–7.28 (ms, 15H, aromatic H), 4.90, 4.88, 4.83 (each AB d, 6H, *J* 11.0, OCH_2Ph), 4.30 (d, 1H, $J_{1,2}$ 7.5, H-1), 4.00–3.90 (overlapping signals, 3H, H-5,6), 3.66 (m, 1H, H-3), 3.53 (s, 3H, OCH_3), 3.41 (m, 1H, H-2), 3.34 (m, 1H, H-4), 1.26–1.05 (ms, 21H, TIPS); ^{13}C NMR ($CDCl_3$): δ 138.98, 138.92, 138.7 (each s, each aromatic C), 128.69, 128.65, 128.62, 128.5, 128.3, 128.2, 128.0, 127.9, 127.8 (each d, each aromatic CH), 104.7 (d, C-1), 84.9, 82.9, 77.8, 76.2 (each d), 76.0, 75.3, 75.0 (each t, each CH_2Ph), 62.7 (t, C-6), 56.9 (q, OCH_3), 18.3, 18.2 (each q, each TIPS CH_3), 12.3 (d, TIPS CH); LRESIMS: Found, *m/z* 643.3; required, 643.9 $[M+Na]^+$.

3.1.4. Methyl 2,3,4-tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranoside (2c)

Treatment of **1c** (4 g, 20 mmol) as described for **1a** gave **2c** (6.5 g, 51%); $[\alpha]_D$ 25.5 (*c* 0.05, $CHCl_3$); FTIR (KBr): 3056, 2864, 1496, 1363, 1324, 970, 882, 790, 734, 696 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.38–7.24 (multiple signals, 15H, each aromatic H), 4.79 (AB d, 2H, *J* 11.0, OCH_2Ph), 4.72 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.71–4.64 (overlapping signals, 3H, OCH_2Ph, H-1), 3.95 (dd, 1H, $J_{2,3}$ 2.0, $J_{3,4}$ 11.0, H-3), 3.93–3.87 (overlapping signals, 3H, H-4,6a,6b), 3.76 (dd, 1H, $J_{1,2}$ 2.5, H-2), 3.59 (dd, 1H, *J* 5.5, *J* 7.0, H-5), 3.31 (s, 3H, OMe), 1.12–1.04 (multiple signals, 21H, TIPS); ^{13}C NMR ($CDCl_3$): δ 138.68, 138.61, 138.4 (each s, each aromatic C), 128.3, 128.2, 127.9, 127.67, 128.63, 127.5, 127.4 (each d, each aromatic CH), 98.5 (d, C-1), 80.3, 76.7, 74.9, 73.3 (each d), 75.1, 72.5, 72.1 (each t, each CH_2Ph), 63.2 (t, C-6), 54.4 (q, OMe), 18.0, 17.9 (each q, each TIPS CH_3), 12.3 (each d, each TIPS CH_2); LRESIMS: Found, *m/z* 638.5; required, 638.9 $[M+H_2O]^+$.

3.1.5. Methyl 2,3,4-tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-galactopyranoside (2d)

Treatment of **1d** (4.0 g, 20.0 mmol) as described for **1a** gave **2d** (6.4 g, 50%); $[\alpha]_D$ 20.6 (*c* 0.07, $CHCl_3$); FTIR (KBr): 3030, 2865, 1496, 1454, 1350, 1194, 1054, 882, 793, 734, 696 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.41–7.22 (multiple signals, 15H, each aromatic H), 4.82 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.71 (AB d, 2H, *J* 11.5, OCH_2Ph), 4.77 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.68 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.04 (dd, 1H, $J_{2,3}$ 10.0, H-2), 3.95–3.92 (overlapping signals, 2H, H-3,5), 3.74–3.64 (overlapping signals, 3H, H-4,6), 3.36 (s, 3H, OMe), 1.12–0.86 (multiple signals, 21H, TIPS); ^{13}C NMR ($CDCl_3$): δ 137.9, 137.7, 137.5 (each s, each aromatic C), 127.33, 127.28, 127.22, 127.15, 127.06, 126.62, 126.48, 126.45 (each d, each aromatic CH), 97.6 (d, C-1), 78.1, 75.4, 74.0, 70.1 (each d), 73.7, 72.5, 72.2 (each t, each CH_2Ph), 61.4 (t, C-6), 54.1 (q, OMe), 16.94, 16.93 (each q, each TIPS CH_3), 10.8 (each d, each TIPS CH_2); LRESIMS: Found, *m/z* 638.5; required, 638.9 $[M+H_2O]^+$.

3.1.6. Methyl 2,3,4-tri-*O*-benzyl-6-*O*-lauroyl-α-D-glucopyranoside (3a)

Compound **2a** (3.0 g, 4.8 mmol) was dissolved in anhyd THF (80 mL), and the solution was cooled to 0 °C. Tetrabutylammonium fluoride (1 g, 4 mmol) was added, and the solution was allowed to warm to room temperature and stirred for 1 h.[25] It was then concentrated in vacuo, and approximately 1 mmol of the resulting 6-OH residue was dissolved in anhyd pyridine (25 mL). 4-Dimethylaminopyridine (DMAP, catalytic amt) and lauroyl chloride (0.29 mL, 1.22 mmol) were added, and the solution was allowed to stir at room temperature for 24 h.[26] It was then concentrated under reduced pressure, and the resulting benzylated ester derivative was purified by chromatography (petroleum ether–EtOAc) to give **3a** (0.47 g, 72%); $[\alpha]_D$ 7.5 (*c* 0.02, $CHCl_3$); FTIR (KBr): 2924, 2853, 1738, 1603, 1502, 1454, 1249, 1072 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.35–7.26 (ms, 15H, aromatic H), 4.92, (AB d, 2H, *J* 10.5, OCH_2Ph), 4.72, (AB d, 2H, *J* 10.5, OCH_2Ph), 4.64 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.59 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.27 (d, 2H, $J_{5,6}$ 3.5, H-6a,6b), 4.01 (apt t, 1H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.0, H-3), 3.82 (d apt t, 1H, $J_{4,5}$ 10.0, H-5), 3.53 (dd, 1H, H-2), 3.48 (apt t, 1H, H-4) 3.37 (s, 3H, OCH_3), 2.35 (m, 2H, aliphatic $OCOCH_2C_{10}H_{21}$), 1.61 (m, 2H, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.28–1.24 (ms, 16H, aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.87 (m, 3H, aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 171.1 (s, C=O), 138.6, 138.1, 137.9 (each s, each aromatic C), 128.5, 128.48, 128.46, 128.1, 128.03, 127.98, 127.90, 127.7 (each d, each aromatic CH), 98.0 (d, C-1), 88.0, 79.9, 77.6, 68.6 (each d), 75.8, 75.1, 73.4 (each t, each CH_2Ph), 60.4 (t, C-6), 55.2 (q, OCH_3), 34.2, 31.9, 29.8, 29.6, 29.5, 29.3, 29.2, 24.9, 22.7, 21.1 (each t, each aliphatic CH_2), 14.2 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 669.39; required, 669.85 $[M+Na]^+$. Anal. Calcd for $C_{40}H_{54}O_7$: C, 74.27; H, 8.41. Found: C, 73.98; H, 8.30.

3.1.7. Methyl 2,3,4-tri-*O*-benzyl-6-*O*-lauroyl-β-D-glucopyranoside (3b)

Treatment of **2b** (3.0 g, 4.8 mmol) as described for **2a** gave **3b** (2.2 g, 70%); $[\alpha]_D$ 8.3 (*c* 0.03, $CHCl_3$); FTIR (KBr): 2924, 2853, 1739, 1497, 1454, 1356, 1151, 1070, 735 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.36–7.24 (ms, 15H, aromatic H), 4.87, 4.84, 4.72 (each AB d, 6H, *J* 10.5, OCH_2Ph), 4.37 (d, 2H, $J_{5,6}$ 11.5, H-6a,6b), 4.31 (d, 1H, $J_{1,2}$ 8.0, H-1), 4.25 (m, 1H, H-5), 3.67 (apt t, 1H, $J_{2,3}$ 8.5, $J_{3,4}$ 8.5, H-3), 3.56 (s, 3H, OCH_3), 3.54 (m, 1H, H-4), 3.43 (dd, 1H, H-2), 2.32 (m, 2H, aliphatic $OCOCH_2C_{10}H_{21}$), 1.62 (m, 2H, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.26–1.24 (ms, 16H, each aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 3H, *J* 6.0, *J* 7.0, aliphatic $OCOC_{11}H_{23}CH_3$); ^{13}C NMR ($CDCl_3$): δ 173.6 (s, C=O), 138.43, 138.42, 137.8 (each s, each aromatic C), 128.8, 128.5, 128.4, 128.38, 128.34, 128.26, 128.11, 128.07, 127.97, 127.92, 127.8, 127.7, 127.69, 127.64, 127.5 (each d, each aromatic CH), 104.7 (d, C-1), 84.6, 82.3, 77.6, 72.9 (each d), 75.7, 75.1, 74.8 (each t, each OCH_2Ph), 62.9 (t, C-6), 57.1 (q, OCH_3), 34.2, 31.9, 29.6, 29.5, 29.3, 29.2, 29.1, 24.9, 24.7, 22.6 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 669.2; required, 669.9 $[M+Na]^+$. Anal. Calcd for $C_{40}H_{54}O_7$: C, 74.27; H, 8.41. Found: C, 73.91; H, 8.79.

3.1.8. Methyl 2,3,4-tri-*O*-benzyl-6-*O*lauroyl-α-D-mannopyranoside (3c)

Treatment of **2c** (6.2 g, 10.0 mmol) as described for **2a** gave **3c** (4.1 g, 64%); $[\alpha]_D$ 23.3 (*c* 0.04, $CHCl_3$); FTIR (KBr): 3031, 2924, 2853, 1737, 1496, 1454, 1362, 1066, 1027, 970, 909, 735, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.38–7.25 (multiple signals, 15H, each aromatic H), 4.77 (AB d, 2H, *J* 10.5, OCH_2Ph), 4.74 (d, 1H, $J_{1,2}$ 2.0, H-1), 4.72 (AB d, 2H, *J* 12.5, OCH_2Ph), 4.61 (s, 2H, OCH_2Ph), 4.38 (dd, 1H, $J_{5,6a}$ 2.5, $J_{6a,6b}$ 12.0, H-6a), 4.33 (dd, 1H, $J_{5,6b}$ 5.0, H-6b), 3.94–3.88 (overlapping signals, 2H, H-3,4), 3.78 (dd, 1H, $J_{2,3}$ 2.5, H-2), 3.76 (m, 1H, H-5), 3.31 (s, 3H, OMe), 2.32 (t, 2H, *J* 7.5, *J* 7.5, aliphatic $OCOCH_2C_{10}H_{21}$), 1.61 (m, 2H, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.31–1.54 (ms, 16H, aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.91–0.86 (m, 3H, aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 173.7, (s, C=O), 138.32, 138.21, 138.17 (each s, each aromatic *C*), 128.4, 128.38, 128.33, 128.05, 127.90, 127.76, 127.63, 127.23 (each d, each aromatic CH), 98.9 (d, C-1), 75.2, 74.6, 74.4, 69.9 (each d), 80.1, 72.6, 72.1 (each t, each CH_2Ph), 63.3 (t, C-6), 54.8 (q, OCH_3), 34.2, 33.9, 31.9, 29.61, 29.48, 29.44, 29.33, 29.27, 29.17, 29.07, 24.9, 24.7, 23.8, 22.7, 21.1 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 664.6; required, 664.9 $[M+H_2O]^+$. Anal. Calcd for $C_{40}H_{54}O_7$: C, 74.27; H, 8.41. Found: C, 74.35; H, 8.25.

3.1.9. Methyl 2,3,4-tri-*O*-benzyl-6-*O*lauroyl-α-D-galactopyranoside (3d)

Treatment of **2d** (5.7 g, 9.2 mmol) as described for **2a** gave **3d** (3.6 g, 60%); $[\alpha]_D$ 27.8 (*c* 0.09, $CHCl_3$); FTIR (KBr): 3030, 2924, 2853, 1738, 1496, 1454, 1350, 1099, 1049, 735, 696 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.41–7.23 (multiple signals, 15H, each aromatic H), 4.83 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.81 (AB d, 2H, *J* 11.5, OCH_2Ph), 4.77 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.68 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.16 (dd, 1H, *J* 7.5, *J* 11.5, H-4), 4.07–4.03 (overlapping signals, 2H, H-2,5), 3.94 (dd, 1H, *J* 3.0, *J* 10.0 H-6a), 3.86–3.84 (overlapping signals, 2H, H-3,6b), 3.35 (s, 3H, OMe), 2.23 (m, 2H, aliphatic $OCOCH_2C_{10}H_{21}$), 1.57 (m, 2H, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.31–1.18 (ms, 16H, aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 3H, *J* 6.5, *J* 7.0, aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 173.4 (s, C=O), 138.7, 138.4, 138.2 (each s, each aromatic C), 128.42, 128.36, 128.32, 128.11, 127.90, 127.75, 127.59, 127.51, 127.21 (each d, each aromatic CH), 98.7 (d, C-1), 78.9, 76.3, 74.9, 68.4 (each d), 74.6, 73.63, 73.54 (each t, each CH_2Ph), 63.3 (t, C-6), 55.3 (q, OCH_3), 34.1, 33.8, 31.9, 29.359, 29.45, 29.32, 29.26, 29.12, 24.9, 24.8, 22.7 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 664.6; required, 664.9 $[M+H_2O]^+$. Anal. Calcd for $C_{40}H_{54}O_7$: C, 74.27; H, 8.41. Found: C, 74.67; H, 8.68.

3.1.10. Methyl 6-*O*-lauroyl-α-D-glucopyranoside (4a)

Compound **3a** (0.34 g, 0.2 mmol) was dissolved in EtOH (1 mL), and Pd/C (0.1 g) was added. The mixture was allowed to shake under hydrogen atmosphere of 2 psi until all protecting groups had been removed, as shown by TLC, to yield **4a**. The suspension was filtered and concentrated in vacuo[27] to give **4a** (0.17 g, 86%); $[\alpha]_D$ 19 (*c* 0.02, $CHCl_3$); FTIR (KBr): 3734, 3445, 2955, 2924, 2850, 2359, 2341, 1728 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.75 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.33 (m, 2H, H-6), 3.75–3.73 (overlapping signals, 2H, H-3,5), 3.35 (apt t, 1H, $J_{3,4}$ 9.5, $J_{4,5}$ 9.5, H-4), 3.54 (dd, 1H, $J_{2,3}$ 9.5, H-2), 3.41 (s, 3H, OMe), 2.35 (t, 2H, *J* 7.5, aliphatic $OCOCH_2C_{10}H_{21}$), 1.63 (m, 2H, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.38–1.23 (ms, 16H, aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 3H, *J* 7.0, aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 174.2 (s, C=O), 99.4 (d, C-1), 74.1, 71.9, 70.4, 69.8 (each d), 63.5 (t, C-6), 55.2 (q, OCH_3), 34.2, 31.9, 29.66, 29.64, 29.5, 29.4, 29.3, 29.2, 24.9, 22.7 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 399.3; required, 399.5 $[M+Na]^+$. Anal. Calcd for $C_{19}H_{36}O_7$: C, 60.61; H, 9.64. Found: C, 60.69; H, 9.83.

3.1.11. Methyl 6-*O*-lauroyl-β-D-glucopyranoside (4b)

Treatment of **3b** (2.0 g, 3.0 mmol) as described for **3a** gave **4b** (0.86 g, 75%); $[\alpha]_D$ −25.5 (*c* 0.05, $CHCl_3$); FTIR (KBr): 3421, 2921, 1744, 1703, 1016 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.40 (d, 1H, $J_{1,2}$ 11.5, H-1), 4.28 (dd, 1H, $J_{2,3}$ 6.0, H-2), 4.21 (d, 2H, $J_{5,6}$ 7.5, H-6), 3.54 (s, 3H, OCH_3), 3.49 (m, 1H, H-3), 3.39–3.31 (overlapping signals, 2H, H-4,5), 2.34 (m, 2H, aliphatic $OCOCH_2C_{10}H_{21}$), 2.02 (s, 3H, OH), 1.62 (m, 2H, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.28–1.26 (ms, 16H, aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 3H, *J* 6.5, aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 174.2 (s, C=O), 103.6 (d, C-1), 76.5, 73.9, 73.4, 70.3 (each d), 63.6 (t, C-6), 57.0 (q, OCH_3), 34.2, 31.9, 29.61, 29.60, 29.5, 29.3, 29.2, 29.1, 24.9, 22.7 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 399.1; required, 399.5 $[M+Na]^+$. Anal. Calcd for $C_{19}H_{36}O_7$: C, 60.61; H, 9.64. Found: C, 60.25; H, 9.91.

3.1.12. Methyl 6-*O*-lauroyl-α-D-mannopyranoside (4c)

Treatment of **3c** (3.3 g, 5.0 mmol) as described for **3a** gave **4c** (1.4 g, 75%); $[\alpha]_D$ 33.3 (*c* 0.01, $CHCl_3$); FTIR (KBr): 3421, 2923, 1736, 1466, 1197, 1057 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.70 (s, 1H, H-1), 4.45 (br s, 1H, OH), 4.36 (d, 2H, *J* 4.0, H-6), 3.96–3.92 (overlapping signals, 2H, OH, H-2), 3.78 (dd, 1H, $J_{2,3}$ 2.5, $J_{3,4}$ 9.0, H-3), 3.71 (m, 1H, H-5), 3.62 (apt t, 1H, $J_{4,5}$ 9.5, H-4) 3.36 (s, 3H, OMe), 2.35 (t, 2H, *J* 7.5, *J* 7.5, aliphatic $OCOCH_2C_{10}H_{21}$), 1.61 (m, 2H, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.29–1.25 (ms, 16H, aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 3H, *J* 6.5, *J* 7.0, aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 174.7 (s, C=O), 100.9 (d, C-1), 71.5, 70.5, 70.4, 67.7 (each d), 63.9 (t, C-6), 54.9 (q, OCH_3), 34.2, 31.9, 29.7, 29.6, 29.5, 29.4, 29.36, 29.34, 29.19, 24.9, 22.7 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 377.3; required, 377.5 $[M+H]^+$. Anal. Calcd for $C_{19}H_{36}O_7$: C, 60.61; H, 9.64. Found: C, 60.71; H, 9.53.

3.1.13. Methyl 6-*O*-lauroyl-α-D-galactopyranoside (4d)

Treatment of **3d** (2.8 g, 4.4 mmol) as described for **3a** gave **4d** (1.43 g, 86%); $[\alpha]_D$ 56.25 (*c* 0.01, $CHCl_3$); FTIR (KBr): 3250, 2918, 1741, 1467, 1194, 1025 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.63 (apt t, 1H, *J* 6.5, *J* 5.0, OH-3), 4.57 (d, 1H, *J* 6.5, OH-2), 4.55 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.13 (dd, 1H, $J_{5,6a}$ 8.0, $J_{6a,6b}$ 11.5, H-6a), 4.07 (dd, 1H, $J_{5,6b}$ 4.0, H-6b), 3.75 (dd, 1H, H-5), 3.68 (apt t, 1H, $J_{3,4}$ 3.5, $J_{4,5}$ 3.0, H-4), 3.58 (ddd, 1H, $J_{2,3}$ 10.0, $J_{2,OH}$ 16.5, H-2), 3.52 (m, 1H, H-3), 3.24 (s, 3H, OMe), 2.28 (t, 2H, *J* 7.5, aliphatic $OCOCH_2C_{10}H_{21}$), 1.63 (t, 2H, *J* 7.0, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.28–1.23 (ms, 16H, aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.85 (t, 3H, *J* 7.0, aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 178.2 (s, C=O), 104.8 (d, C-1), 74.9, 74.1, 73.7, 73.1 (each d), 68.8 (t, C-6), 59.8 (q, OCH_3), 38.9, 36.5, 34.24, 34.10, 33.97, 33.93, 33.75, 29.5, 27.3, (each t, each aliphatic CH_2), 18.9 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 399.3; required, 399.5 $[M+Na]^+$. Anal. Calcd for $C_{19}H_{36}O_7$: C, 60.61; H, 9.64. Found: C, 60.60; H, 9.88.

3.1.14. Methyl 2,3,4-tri-*O*-benzyl-6-*O*octanoyl-α-D-glucopyranoside (5a)

Compound **2a** (5.0 g, 8.5 mmol) was dissolved in anhyd THF (150 mL), and the solution was cooled to 0 °C. Tetrabutylammonium fluoride (2.2 g, 8.5 mmol) was added, and the solution was warmed to room temperature and stirred for 1 h.[25] The mixture was then concentrated in vacuo, and the resulting 6-OH residue was dissolved in anhyd pyridine (100 mL), 4-Dimethylaminopyridine (DMAP, catalytic amt) and octanoyl chloride (2.9 mL, 17 mmol) were added, and the mixture was stirred at room temperature for 24 h.[26] The solution was then concentrated under reduced pressure and purified by chromatography (petroleum ether–EtOAc) to give **5a** (3.9 g, 63%); $[\alpha]_D$ 20.8 (*c* 0.07, $CHCl_3$); FTIR (KBr): 2927, 1738, 1497, 1454, 1360, 1163, 1093, 738, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.37–7.26 (ms, 15H, aromatic H), 4.93, (AB d,

2H, *J* 10.5, OCH_2Ph), 4.74, (AB d, 2H, *J* 12.0, OCH_2Ph), 4.73 (AB d, 2H, *J* 10.5, OCH_2Ph), 4.60 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.28 (d, 2H, $J_{5,6}$ 3.0, H-6), 4.01 (apt t, 1H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.5, H-3), 3.81 (m, 1H, H-5), 3.54 (dd, 1H, H-2), 3.48 (dd, 1H, $J_{4,5}$ 10.5, H-4), 3.37 (s, 3H, OCH_3), 2.31 (m, 2H, aliphatic $OCOCH_2C_6H_{13}$), 1.62 (m, 2H, aliphatic $OCH_2CH_2C_5H_{11}$), 1.30–1.05 (ms, 8H, aliphatic $OC_2H_4C_4H_8CH_3$), 0.87 (m, 3H, aliphatic $OC_6H_{12}CH_3$); ^{13}C NMR ($CDCl_3$): δ 173.8 (s, C=O), 138.8, 138.3, 138.1 (each s, each aromatic C), 128.7, 128.6, 128.3, 128.29, 128.27, 128.3, 128.25, 128.20, 128.1 127.9 (each d, each aromatic CH), 98.3 (d, C-1), 82.2, 80.2, 77.8, 68.9 (each d), 76.1, 75.3, 73.6 (each t, each OCH_2Ph), 63.1 (t, C-6), 55.4 (q, OCH_3), 34.4, 31.9, 29.2, 25.0, 22.8, 17.9 (each t, each aliphatic CH_2), 14.3 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 613.4; required, 613.7 $[M+Na]^+$. Anal. Calcd for $C_{36}H_{46}O_7$: C, 73.19; H, 7.85. Found: C, 73.25; H, 7.61.

3.1.15. Methyl 6-*O*-octanoyl-α-D-glucopyranoside (6a)

Treatment of **5a** (3.6 g, 6.2 mmol) as described for **3a** gave **6a** (1.44 g, 73%); $[\alpha]_D$ 27.9 (*c* 0.4, $CHCl_3$); FTIR (KBr): 3388, 2922, 1712, 1465, 1193, 1106, 724 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 5.82 (s, 3H, each O*H*), 4.76 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.35 (d, 2H, $J_{5,6}$ 4.0, H-6), 3.78–3.72 (overlapping signals, 2H, H-3,5), 3.54 (dd, 1H, $J_{2,3}$ 9.5, H-2), 3.41 (s, 3H, OCH_3), 3.36 (dd, 1H, $J_{3,4}$ 9.5, $J_{4,5}$ 10.0, H-4), 2.35 (m, 2H, aliphatic $COCH_2C_6H_{13}$) 1.64 (t, 2H, *J* 7.0, aliphatic $COCH_2CH_2C_5H_{11}$), 1.31–1.05 (ms, 8H, aliphatic $COC_2H_4C_4H_8CH_3$), 0.88 (t, 3H, *J* 5.5, *J* 7.0, aliphatic $COC_6H_{12}CH_3$); ^{13}C NMR ($CDCl_3$): δ 179.5 (s, C=O), 99.4 (d, C-1), 74.1, 72.0, 69.7, 70.3 (each d), 63.4 (t, C-6), 55.3 (q, OCH_3), 34.1, 31.7, 31.6, 29.9, 28.9, 24.8 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 343.1; required, 343.4 $[M+Na]^+$. Anal. Calcd for $C_{15}H_{28}O_7$: C, 56.23; H, 8.81. Found: C, 56.47; H, 8.73.

3.1.16. Methyl 2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-triisopropylsilyl-α-D-glucopyranoside (7a)

A solution of **1a** (5.0 g, 25.0 mmol) in anhyd DMF (120 mL) was treated with chlorotriisopropylsilane (TIPSCl, 15 mL, 75 mmol) and imidazole (5 g, 75 mmol), and allowed to stir at room temperature for 24 h. The crude TIPS-protected intermediate was then concentrated in vacuo, and the resulting residue was dissolved in EtOAc. The organic extract was then washed with 10% HCl and water, followed by satd aq $NaHCO_3$ and finally satd aq NaCl. It was then dried over anhyd $MgSO_4$ and concentrated under reduced pressure.[23] The TIPS-protected crude product was then split into two parts, and half was dissolved in a mixture of anhyd DMF (30 mL) and anhyd THF (20 mL). This solution was then added dropwise at 0 °C to a suspension of NaH (2.5 g, 62.5 mmol) in anhyd DMF (10 mL) and anhyd THF (7 mL), *p*-methoxybenzyl chloride (17 mL, 125 mmol) and tetrabutylammonium iodide (18.5 g, 50 mmol). The mixture was stirred at ~10 °C for 30 min and then allowed to warm to room temperature and stirred for 24 h. MeOH (50 mL) was added to quench the reaction, and the mixture was stirred for 1 h. The solution was then concentrated under diminished pressure and dissolved in EtOAc. The organic extract was washed with water, dried over anhyd $MgSO_4$ and concentrated in vacuo.[28] The resulting residue was purified by chromatography (petroleum ether–EtOAc) to give **7a** (5.15 g, 59%); $[\alpha]_D$ 11.6 (*c* 0.05, $CHCl_3$); FTIR (KBr): 3479, 2936, 2864, 1464, 1421, 1360, 1302, 883, 820 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.34–6.73 (ms, 12H, aromatic H), 4.88 (AB d, 2H, *J* 10.5 OCH_2Ph), 4.78 (d, 1H, $J_{1,2}$ 5.0, H-1), 4.75, 4.71 (each AB d, 2H, *J* 12.0 OCH_2Ph), 4.63 (m, 1H, H-2), 3.99 (apt t, 1H, $J_{3,4}$ 9.0, $J_{4,5}$ 9.0, H-4), 3.89 (m, 2H, H6), 3.77 (m, 9H, each $PhOCH_3$), 3.57–3.49 (overlapping signals, 2H, H-3,5), 3.39 (s, 3H, OCH_3), 1.28 (m, 3H, each TIPS C*H*), 1.16–1.06 (ms, 18H, each TIPS CH_3); ^{13}C NMR ($CDCl_3$): δ 159.6, 159.5, 159.4, 131.6, 131.4, 131.0 (each s, each aromatic C), 129.99, 129.93, 129.8, 114.13, 114.08, 113.6 (each d, each aromatic CH), 98.1 (d, C-1), 82.2, 80.2, 77.8, 72.1 (each d), 75.8, 74.9, 73.2 (each t, each OCH_2Ph), 63.1 (t, C-6), 55.47, 55.40, 55.36 (each q, each $PhOCH_3$), 55.0 (q, OCH_3), 18.27, 18.25 (each q, each TIPS CH_3), 12.3 (d, each TIPS CH); LRESIMS: Found, *m/z* 733.3; required, 733.9 $[M+Na]^+$.

3.1.17. Methyl 2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-triisopropylsilyl-β-D-glucopyranoside (7b)

Treatment of **1b** (4.5 g, 23.17 mmol) as described for **1a** gave **7b** (10.1 g, 61%); $[\alpha]_D$ 4.8 (*c* 0.05, $CHCl_3$); FTIR (KBr): 2939, 1586, 1464, 883, 821, 760, 683 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.30–6.84 (ms, 12H, aromatic H), 4.85, 4.80, 4.73 (each AB d, 2H, *J* 10.5, OCH_2Ph), 4.27 (d, 1H, $J_{1,2}$ 7.5, H-1), 3.95 (m, 1H, H-6a), 3.87 (dd, 1H, $J_{4,5}$ 11.0, $J_{5,6}$ 4.5, H-5), 3.78 (m, 9H, $PhOCH_3$), 3.59 (m, 1H, H-3), 3.53 (s, 3H, OCH_3) 3.36 (apt t, 1H, $J_{2,3}$ 9.0, H-2), 3.29–3.24 (overlapping signals, 2H, H-4,6b), 1.10–1.04 (ms, 21H, TIPS); ^{13}C NMR ($CDCl_3$): δ 159.5, 159.4, 131.2, 131.1, 130.9, (each s, each aromatic C), 129.9, 129.8, 128.7, 114.1, 114.04, 114.01 (each d, each aromatic CH), 104.7 (d, C-1), 84.7, 82.6, 77.5, 76.2 (each d), 75.7, 74.9, 74.7 (each t, each OCH_2PH), 62.7 (t, C-6), 56.8 (q, OCH_3), 55.5 (each q, each $PhOCH_3$), 18.3, 18.2 (each q, each TIPS CH_3), 12.2 (d, each TIPS CH); LRESIMS: Found, *m/z* 733.3; required, 733.9 $[M+Na]^+$.

3.1.18. Methyl 2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-dodecanyl-α-D-glucopyranoside (8a)

Compound **7a** (4.0 g, 5.5 mmol) was dissolved in anhyd THF (100 mL), and the solution was cooled to 0 °C. Tetrabutylammonium fluoride (1.4 g, 5.5 mmol) was added, and the solution was allowed to warm to room temperature and stirred for 1 h.[25] The mixture was then concentrated in vacuo, and the resulting 6-OH residue was dissolved in anhyd DMF (100 mL). 1-Chlorododecane (1.8 mL, 11 mmol) was added, and the solution was cooled to 0 °C before NaH (0.11 g, 2.75 mmol) was added portionwise. The mixture was then allowed to warm to room temperature and was stirred for 24 h. MeOH (50 mL) was added to quench the reaction, and the mixture was stirred for 1 h.[29] The crude PMB-protected ether was then concentrated under diminished pressure and purified by chromatography (petroleum ether–EtOAc) to give **8a** (1.89 g, 50%); $[\alpha]_D$ −8.6 (*c* 0.06, $CHCl_3$); FTIR (KBr): 2924, 2854, 1613, 1586, 1464, 1359, 1301, 1248, 1172, 1037, 820 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 6.85–7.30 (ms, 12H, aromatic H), 4.92 (d, 1H, $J_{1,2}$ 10.5, H-1), 4.85 (AB d, 2H, *J* 10.5, OCH_2PhOCH_3), 4.74 (dd, 1H, $J_{2,3}$ 9.5, H-2), 4.69, (AB d, 2H, *J* 10.5, OCH_2PhOCH_3), 4.60 (AB d, 2H, *J* 11.5 OCH_2PhOCH_3), 4.55 (apt t, 1H, $J_{3,4}$ 9.5, H-3), 3.95 (m, 1H, H-5), 3.80 (s, 9H, each $PhOCH_3$), 3.53–3.37 (overlapping signals, 3H, H-4,6a,6b), 3.36 (s, 3H, OCH_3), 1.60 (m, 2H, aliphatic $CH_2C_{11}H_{23}$), 1.30–1.25 (ms, 20H, aliphatic $CH_2C_{10}H_{20}CH_3$), 0.89 (t, 3H, *J* 7.0, aliphatic $C_{11}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 159.6, 159.5, 159.4, 131.3, 131.0, 130.6 (each s, each aromatic C), 130.0, 129.8, 129.6, 114.07, 114.05, 114.03 (each d, each aromatic CH), 98.5 (d, C-1), 82.1, 79.8, 77.7, 70.2 (each d), 75.7, 74.9, 73.3 (each t, each OCH_2Ph), 72.0 (t, aliphatic $OCH_2C_{11}H_{23}$), 69.5 (t, C-6), 55.5 (q, $PhOCH_3$), 55.3 (s, OCH_3), 32.2, 29.94, 29.91, 29.89, 29.87, 29.84, 29.7, 29.5, 28.4 (each t, each aliphatic CH_2), 14.4 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 745.5; required, 745.9 $[M+Na]^+$. Anal. Calcd for $C_{43}H_{62}O_9$: C, 71.44; H, 8.64. Found: C, 71.09; H, 8.73.

3.1.19. Methyl 2,3,4-tri-*O*-paramethoxybenzyl-6-*O*-dodecanyl-β-D-glucopyranoside (8b)

Treatment of **7b** (3.2 g, 4.5 mmol) as described for **7a** gave **8b** (0.55 g, 85%); $[\alpha]_D$ 2 (*c* 0.01, $CHCl_3$); FTIR (KBr): 2923, 2851, 1614, 1464.40, 1421, 1359, 1302, 1254, 1173, 1072, 813 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.29–6.84 (ms, 12H, aromatic H), 4.79, 4.75, 4.67 (each AB d, 2H, *J* 10.5, OCH_2Ph), 4.26 (d, 1H, $J_{1,2}$ 7.5, H-1), 3.79–3.58 (overlapping signals, 2H, H-3,5), 3.79 (m, 9H, $PhOCH_3$), 3.68 (m, 2H, H-6a,6b), 3.56 (s, 3H, OCH_3), 3.43–3.39

(overlapping signals, 2H, H-2,4), 1.63 (m, 2H, aliphatic $OCH_2C_{11}H_{23}$), 1.29–1.24 (ms, 20H, aliphatic $OCH_2C_{10}H_{20}CH_3$), 0.88 (t, 3H, *J* 7.0, aliphatic $OC_{11}H_{22}CH_3$); ^{13}C NMR ($CDCl_3$): δ 159.3, 159.2, 159.1, 130.9, 130.8, 130.5 (each s, each aromatic C), 129.8, 129.6, 129.5, 113.8, 113.7 (each d, each aromatic CH), 104.8 (d, C-1), 84.4, 82.1, 77.7, 75.3 (each d), 74.9, 74.6, 74.4 (each t, each OCH_2Ph), 71.9 (t, aliphatic CH_2), 69.7 (t, C-6), 57.1 (q, OCH_3), 55.3, 55.2 (each q, each $PhOCH_3$), 31.9, 29.7, 29.68, 29.65, 29.63, 29.5, 29.4, 26.2, 22.7 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 745.3; required, 745.9 $[M+Na]^+$. Anal. Calcd for $C_{43}H_{62}O_9$: C, 71.44; H, 8.64. Found: C, 71.19; H, 8.70.

3.1.20. Methyl 6-*O*-dodecanyl-α-D-glucopyranoside (9a)

Compound **8a** (1.45 g, 2.0 mmol) was dissolved in 3:1 MeCN–H_2O (21 mL), and cerric ammonium nitrate (8.85 g, 16.16 mmol) was added. The solution was allowed to stir at room temperature for 24 h.[30] It was then concentrated in vacuo, and the residue was purified by chromatography (petroleum ether–EtOAc) to give **9a** (0.53 g, 73%); $[\alpha]_D$ 78.8 (*c* 0.04, $CHCl_3$); FTIR (KBr): 3416, 2919, 2851, 1467, 1372, 1128, 1043, 1019 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.98 (br s, 1H, OH), 4.75 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.34 (br s, 1H, OH), 4.01 (br s, 1H, OH), 3.75 (apt t, 1H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.5, H-3), 3.66 (m, 2H, H-6), 3.54–3.44 (overlapping signals, 3H, H-2,4,5), 3.37 (s, 3H, OCH_3), 1.58 (m, 2H, aliphatic $CH_2C_{11}H_{23}$), 1.28–1.25 (ms, 20H, each aliphatic $CH_2C_{10}H_{20}CH_3$), 0.88 (t, 3H, *J* 6.5, *J* 7.0, aliphatic $C_{11}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 99.7 (d, C-1), 74.5, 72.3, 72.2, 71.2 (each d) 70.6 (t, aliphatic CH_2), 69.5 (t, C-6), 55.4 (q, OCH_3), 32.1, 29.9, 29.88, 29.86, 29.83, 29.7, 29.6, 26.3, 22.9 (each t, each aliphatic CH_2), 14.3 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 385.2; required, 385.5 $[M+Na]^+$. Anal. Calcd for $C_{19}H_{38}O_6$: C, 62.95; H, 10.57. Found: C, 62.60; H, 10.67.

3.1.21. Methyl 6-*O*-dodecanyl-β-D-glucopyranoside (9b)

Treatment of **8b** (0.44 g, 0.6 mmol) as described for **8a** gave **9b** (0.17 g, 76%); $[\alpha]_D$ −1 (*c* 0.03, $CHCl_3$); FTIR (KBr): 3405, 2922, 2850, 1470, 1391, 1128, 1109, 1048 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.20 (d, 1H, $J_{1,2}$ 7.5, H-1), 3.89 (s, 1H, OH), 3.74 (m, 2H, H-6a,6b), 3.66 (m, 1H, H-5), 3.54 (s, 3H, OCH_3), 3.52–3.44 (overlapping signals, 2H, H-3,4), 3.35 (apt t, 1H, $J_{2,3}$ 8.0, H-2), 1.58 (m, 2H, aliphatic $OCH_2C_{11}H_{23}$), 1.28–1.11 (ms, 20H, aliphatic $OCH_2C_{10}H_{20}CH_3$), 0.88 (t, 3H, *J* 6.5, *J* 7.0, aliphatic $OC_{11}H_{22}CH_3$); ^{13}C NMR ($CDCl_3$): δ 103.5 (d, C-1), 76.5, 74.4, 73.4, 72.1, (each d), 71.6 (t, aliphatic CH_2), 70.9 (t, C-6), 57.1 (q, OCH_3), 31.9, 29.7, 29.66, 29.65, 29.58, 29.53, 29.4, 26.0, 22.7 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 385.2; required, 385.5 $[M+Na]^+$. Anal. Calcd for $C_{19}H_{38}O_6$: C, 62.95; H, 10.57. Found: C, 62.83; H, 10.36.

3.1.22. Methyl 2,3-di-*O*-benzyl-4,6-di-*O*-benzylidene-α-D-glucopyranoside (10a)

A solution of **1a** (1.0 g, 5.2 mmol), *p*-toluenesulfonic acid (10 mg) and benzaldehyde dimethylacetal (1.5 mL, 10.3 mmol) in anhyd MeCN (25 mL) was stirred for 24 h at room temperature. Me_3N (0.5 mL) was added to neutralise the solution, and the mixture was then stirred for 1 h. The product was filtered off as a white solid, washed with petroleum ether and dried. The benzylidene-protected intermediate was then dissolved in anhyd DMF (15 mL), and the solution was cooled to 0 °C. NaH (0.74 g, 18.4 mmol) was added slowly, followed by benzyl bromide (2.5 mL, 20 mmol). The mixture was then warmed to room temperature and stirred overnight. MeOH (10 mL) was added to quench the reaction, and the mixture was stirred for a further 1 h.[24] The mixture was then concentrated under diminished pressure and purified by chromatography (petroleum ether–EtOAc) to give **10a** (2.0 g, 95%); $[\alpha]_D$ 0.7 (*c* 0.05, $CHCl_3$); FTIR (KBr): 3063, 3031, 1109, 1088, 735, 692 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.50–7.22 (ms, 15H, each aromatic H), 5.54 (s, 1H, *CH*Ph), 4.85 (AB d, 2H, *J* 4.0, OCH_2Ph), 4.82 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.59 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.26 (dd, 1H, $J_{5,6a}$ 10.0, $J_{6a,6b}$ 4.5, H-6a), 4.05 (apt t, 1H, $J_{2,3}$ 9.0, $J_{3,4}$ 9.0, H-3), 3.83 (m, 1H, H-5), 3.70 (apt t, 1H, $J_{5,6b}$ 10.5, H-6b), 3.62–3.54 (overlapping signals, 2H, H-2,4), 3.39 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$): δ 138.7, 138.1, 137.4 (each s, each aromatic C), 128.89, 128.43, 128.29, 128.20, 128.10, 128.01, 127.90, 127.57, 126.0 (each d, each aromatic CH), 101.2 (d, C-1), 99.2 (d, *CH*Ph), 82.1, 79.2, 78.6, 62.3 (each d), 75.3, 73.8 (each t), 69.1 (t, C-6), 55.3 (q, OCH_3); LRESIMS: Found, *m/z* 463.3; required, 463.5 $[M+H]^+$. Anal. Calcd for $C_{28}H_{30}O_6$: C, 72.71; H, 6.54. Found: C, 72.31; H, 6.56.

3.1.23. Methyl 4,6-di-*O*-lauroyl-α-D-glucopyranoside (12a)

3.1.23.1. Methyl 2,3-di-*O*-benzyl-4,6-di-*O*-lauroyl-α-D-glucopyranoside (11a). Compound **10a** (1.7 g, 3.6 mmol) was dissolved in MeOH (50 mL), and a catalytic amount of TsOH was added. The solution was stirred at room temperature overnight, after which Et_3N (2 mL) was added to quench the reaction.[31] The mixture was concentrated under diminished pressure, and the crude diol residue was dissolved in anhyd pyridine (70 mL). 4-Dimethylaminopyridine (DMAP, catalytic amt) and lauroyl chloride (3.3 mL, 14.4 mmol) were added, and the reaction was stirred at room temperature for 3 h.[26] The solution was then concentrated under diminished pressure, and the crude product was purified by chromatography (petroleum ether–EtOAc) to give **11a** (1.0 g, 38%). FTIR (KBr): 2925, 2853, 1743, 1455, 1360, 1167, 1105, 1045, 734 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.34–7.26 (multiple signal, 10H, each aromatic H), 5.01 (dd, 1H, $J_{3,4}$ 9.5, $J_{4,5}$ 10.0, H-4), 4.78 (AB d, 2H, *J* 11.5, OCH_2Ph), 4.73 (AB d, 2H, *J* 12.0, OCH_2Ph), 4.59 (d, 1H, $J_{1,2}$ 3.5, H-1), 4.15 (dd, 1H, $J_{5,6a}$ 5.5, $J_{6a,6b}$ 12.5, H-6a), 4.04 (dd, 1H, $J_{5,6b}$ 2.0, H-6b), 3.92 (apt t, 1H, $J_{2,3}$ 9.5, H-3), 3.87–3.82 (m, 1H, H-5), 3.59 (dd, 1H, H-2), 2.36–2.27 (m, 4H, each aliphatic $OCOCH_2C_{10}H_{21}$), 1.67–1.56 (m, 4H, each aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.26–1.16 (ms, 32H, each aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 6H, *J* 6.5, *J* 7.0, each aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 173.6, 172.4 (each s, each C=O), 138.4, 137.9 (each s, each aromatic C), 128.51, 128.32, 128.18, 128.05, 127.69, 127.57 (each d, each aromatic CH), 98.2 (d, C-1), 79.51, 79.18, 69.5, 67.7 (each d), 75.4, 73.6 (each t, each CH_2Ph), 62.2 (t, C-6), 55.4 (q, OCH_3), 34.15, 34.03, 33.99, 31.9, 29.62, 29.60, 29.49, 29.44, 29.35, 29.34, 29.28, 29.26, 29.15, 29.13, 29.07, 24.76, 24.70, 22.69 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3).

3.1.23.2. Methyl 4,6-di-*O*-lauroyl-α-D-glucopyranoside (12a). Compound **11a** (0.84 g, 1.14 mmol) was dissolved in EtOH (2.5 mL), and Pd/C (0.3 g) was added. The mixture was allowed to shake under a hydrogen atmosphere of 2 psi until all protecting groups had been removed (TLC) to yield **12a**. The suspension was filtered and concentrated in vacuo[27] to give **12a** (0.47 g, 75%). $[\alpha]_D$ 4.33 (*c* 0.03, $CHCl_3$); FTIR (KBr): 3456, 2918, 2849, 1737, 1701, 1468, 1301, 1240, 1187, 1046 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.87 (dd, 1H, $J_{3,4}$ 9.5, $J_{4,5}$ 10, H-4), 4.82 (d, 1H, $J_{1,2}$ 4.0, H-1), 4.23 (dd, 1H, $J_{5,6b}$ 2.0, $J_{6a,6b}$ 12.0, H-6b), 4.12 (dd, 1H, $J_{5,6a}$ 2.0, H-6a), 3.91 (ddd, 1H, H-5), 3.84 (apt t, 1H, $J_{2,3}$ 9.5, H-3), 3.64 (m, 1H, H-2), 3.44 (s, 3H, OMe), 2.37–2.32 (m, 4H, each aliphatic $OCOCH_2C_{10}H_{21}$), 1.68–1.55 (m, 4H, each aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.30–1.26 (multiple signals, 32H, each aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 6H, *J* 6.5, *J* 7.0, each aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 173.63, 173.58 (each s, each C=O), 99.0 (d, C-1), 72.9, 72.7, 70.3, 67.7 (each d), 62.2 (t, C-6), 55.5 (q, OMe), 34.2, 34.1, 34.0, 31.9, 29.63, 29.61, 29.50, 29.47, 29.45, 29.36, 29.30, 29.27, 29.14, 29.08, 24.84, 24.82, 24.70, 22.70 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found, *m/z* 559.5; required, 559.8 $[M+H]^+$. Anal. Calcd for $C_{31}H_{58}O_8$: C, 66.63; H, 10.46. Found: C, 66.66; H, 10.79.

3.1.24. General procedure for the preparation of pentaerythritol esters

Pentaerythritol **13** (1.0 g, 7.3 mmol), lauroyl chloride (4.8 mL, 21 mmol) and 4-dimethylaminopyridine (DMAP, catalytic amt) were dissolved in anhyd pyridine (50 mL) and stirred at 50 °C for 24 h.[26] The solution was then concentrated in vacuo, and the following mono-*O*-lauroyl **14** and di-*O*-lauroyl **15** products were isolated by chromatography (petroleum ether–EtOAc). A tetra-*O*-lauroyl derivative was also isolated (0.39 g, 6%):

3.1.25. Mono-*O*-lauroyl pentaerythritol (14)

Yield: 0.33 g (14%); FTIR (KBr): 3462, 2914, 2848, 1737, 1712, 1476, 1187, 1038, 1005 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.10 (s, 2H, $CH_2OC{=}O$), 3.80–3.61 (overlapping signals, 9H, 3 × CH_2OH, 3 × OH), 2.34 (t, 2H, *J* 6.0, *J* 7.0, aliphatic $OCOCH_2C_{10}H_{21}$), 1.61 (m, 2H, aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.26 (ms, 16H, aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (m, 3H, aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 175.0 (s, C=O), 62.7, 62.4 (each t, each CH_2O), 45.3 (s, $C(CH_2)_4$), 34.2, 31.9, 29.59, 29.57, 29.44, 29.30, 29.23, 29.15, 24.9, 22.6 (each t, each aliphatic CH_2), 14.1 (q, aliphatic CH_3); LRESIMS: Found *m/z* 341.2; required, 341.45 $[M+Na]^+$. Anal. Calcd for $C_{17}H_{34}O_5$: C, 64.12; H, 10.76. Found: C, 64.08; H, 10.79.

3.1.26. Di-*O*-lauroyl pentaerythritol (15)

Yiled: 1.074 g (29%); FTIR (KBr): 3351, 2915, 2850, 1739, 1701, 1471, 1163, 978, 719 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.12 (s, 4H, each $CH_2OC{=}O$), 3.58 (s, 4H, each CH_2OH), 3.22 (br s, 2H, each OH) 2.34 (t, 4H, *J* 7.5, *J* 7.5, each aliphatic $OCOCH_2C_{10}H_{21}$), 1.62 (t, 4H, *J* 6.5, *J* 6.5, each aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.29–1.26 (ms, 32H, each aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 6H, *J* 6.5, *J* 6.5, each aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 174.4 (s, each C=O), 62.4 (t, each CH_2O), 44.7 (s, $C(CH_2)_4$), 34.2, 31.9, 29.56, 29.29, 29.21, 29.11, 24.9, 22.6 (each t, each aliphatic CH_2), 14.1 (q, each aliphatic CH_3); LRESIMS: Found *m/z* 501.5; required, 501.75 $[M+H]^+$. Anal. Calcd for $C_{29}H_{56}O_6$: C, 69.56; H, 11.27. Found: C, 69.64; H, 11.31.

3.1.27. Tetra-*O*-lauroyl pentaerythritol

Yield: 0.39 g (6%); FTIR (KBr): 2917, 2849, 1735, 1336, 1299, 1250, 1154, 1111, 1002 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 4.11 (s, 8H, each $CH_2OC{=}O$), 2.30 (t, 8H, *J* 7.5, *J* 8.0, each aliphatic $OCOCH_2C_{10}H_{21}$), 1.60 (t, 8H, *J* 6.5, *J* 7.0, each aliphatic $OCOCH_2CH_2C_9H_{19}$), 1.41–1.26 (ms, 64H, each aliphatic $OCOC_2H_4C_8H_{16}CH_3$), 0.88 (t, 12H, *J* 6.5, *J* 7.0, each aliphatic $OCOC_{10}H_{20}CH_3$); ^{13}C NMR ($CDCl_3$): δ 173.2 (s, each C=O), 62.1 (t, each CH_2O), 41.8 (s, $C(CH_2)_4$), 34.1, 31.9, 29.59, 29.45, 29.31, 29.23, 29.11, 24.8, 22.7 (each t, each aliphatic CH_2), 14.1 (each q, each aliphatic CH_3); LRESIMS: Found *m/z* 888.7; required, 888.36 $[M+Na]^+$. Anal. Calcd for $C_{53}H_{100}O_8$: C, 73.56; H, 11.65. Found: C, 73.60; H, 11.58.

3.2. Evaluation of antimicrobial activity

3.2.1. Preparation of bacterial cultures

Bacteria used in this study were *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. 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. 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 35 °C 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.

3.2.2. Antimicrobial activity assay

Stock solutions (100 mmol) of test compounds and standards were prepared in sterile aqueous-alcoholic diluent (1:1 EtOH–distilled H_2O) and stored at −20 °C. Stock solutions were diluted in TSB to obtain initial working concentrations (10 or 20 mmol). Working test compounds and standards were serially diluted in sterile TSB to a final volume of 100 μL within the 96-well plate. Freshly prepared inoculum (100 μL) of the organism under study was added to each appropriate well. The final concentration of each microorganism in each well was ~5×10^5 cfu/mL, and the concentration range of chemical compounds was 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 EtOH used to dissolve the test compound, thereby assessing any activity of the alcohol. The 96-well plates were incubated at 35 °C for 18 h in a microtiterplate reader (PowerWave microplate Spectrophotometer, BioTek), 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. 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. Subtraction of the absorbance of the negative control eliminated interferences due to variation in the media.

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1.2 Congress & Conferences

- FEMS 2009. 3rd Congress of European Microbiologists, Gothenburg-Sweden, June 28-July 2, 2009. Poster: In vitro antimicrobial activity and mechanisms of action of novel carbohydrate fatty acid derivatives against *Staphylococcus aureus* and MRSA.

P. Nobmann, J. Dunne, G. Henehan and P. Bourke

- Food Micro conference 2008, Aberdeen-Scotland, 1st – 4th September 2008. Poster: Synthesis and antimicrobial activity of novel carbohydrate fatty acid derivatives on food pathogens and food spoilage microorganisms.

P. Nobmann, A. Smith, J. Dunne, G. Henehan and P. Bourke

- 60th Irish Universities Chemistry Research Colloquium, University College Cork, 11th-13th June 2008. Poster: Synthesis, antimicrobial efficacy and structure/activity relationship of novel carbohydrate fatty acid derivatives.

P. Nobmann, A. Smith, P. Bourke, J. Dunne and G. Henehan

- 59th Irish Universities Chemistry Research Colloquium, Dublin City University, 14th-15th June 2007. Poster: Synthesis of novel carbohydrate fatty acid derivatives and the relationship of structure with anti-microbial efficacy.

A. Smith, P. Nobmann, J. Dunne, P. Bourke and G. Henehan

FEMS 2009. 3rd Congress of European Microbiologists, Gothenburg-Sweden, June 28-July 2, 2009

In vitro antimicrobial activity and mechanisms of action of novel carbohydrate fatty acid derivatives against *S. aureus* and MRSA

Patricia Nobmann, Julie Dunne, Gary Henehan and Paula Bourke

School of Food Science and Environmental Health, Dublin Institute of Technology (DIT). Cathal Brugha St., Dublin 1, Ireland
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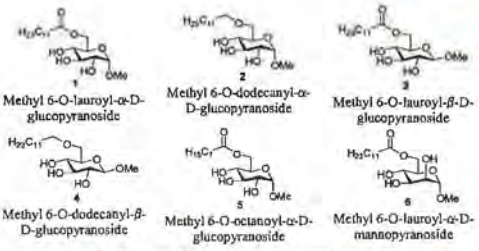
INTRODUCTION

- MRSA and antibiotic resistance is a global concern. Consequently, new antistaphylococcal agents are needed. Unlike antibiotics, fatty acids and their derivatives have modes of action that appear to be non-specific and development of resistance to these compounds has not been reported [i].
- The antimicrobial activity of fatty acids and derivatives has been well documented [ii]. Previous work in this laboratory showed that novel carbohydrate fatty acid (CFA) derivatives had antimicrobial activity against foodborne pathogens and spoilage microorganisms [iii].
- It has been reported[iv] that the half life of monolaurin in cultures of *S. aureus* was *ca.* 5 min, attributed to its cleavage by cellular esterases, which raised the question as to whether the **ester conjugate** or **free fatty acid** derived from hydrolysis of the ester, was responsible for the antimicrobial activity.
- In contrast to **fatty acid ester conjugates**, corresponding **ether conjugates** should then remain antimicrobial for a longer period of time[v], assuming the fatty acid component does not require release by esterases for efficacy.

AIMS

This study was designed to investigate and compare the *in vitro* antimicrobial activity of a range of pure, novel CFA esters and fatty acid ethers, using commercial fatty acids and monoglycerides as control compounds against *S. aureus* and MRSA strains. The resulting findings enabled insights into structure/activity relationships and the mechanism of action of these compounds. The role of the carbohydrate moiety in the antibacterial activity of these compounds is discussed.

SYNTHESIS & METHODOLOGY



Structures of the novel carbohydrate fatty acid derivatives synthesised and investigated [vi].

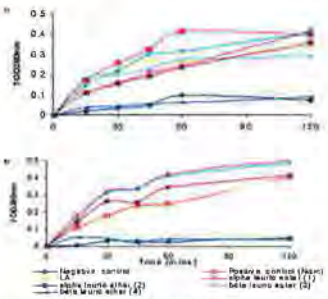
Minimum Inhibitory Concentration (MIC) using an absorbance based broth microdilution method.

Leakage of intracellular material absorbing at 260nm was examined following exposure to CFA derivatives at 4x MIC to assess membrane damage.

RESULTS AND DISCUSSION

Microorganism	FA		MG		Carbohydrate fatty acid derivatives					
	LA	CA	ML	MC	1	2	3	4	5	6
S. aureus ATCC25923	[illegible]	10	0.04	5	0.31	0.04	0.04	2.5	2.5	0.04
S. aureus NCTC1803	[illegible]	10	0.04	2.5	0.31	0.04	0.08	1.25	1.25	0.04
S. aureus ATCC33591	[illegible]	10	0.04	2.5	1.25	0.04	0.04	2.5	5	0.04
S. aureus ATCC33592	[illegible]	20	0.04	2.5	0.08	0.08	0.04	5	2.5	0.16
S. aureus ATCC43300	[illegible]	10	0.08	2.5	0.08	0.08	0.04	10	2.5	0.08

For each analysis the **MIC** was recorded as the concentration (**mM**) that resulted in total inhibition of all replicates at 37°C after 24 hours. 1. α-lauric ester; 2. α-lauric ether; 3. β-lauric ester; 4. β-lauric ether; 5. α-caprylic ester; 6. α -lauric ester of mannose



Increase in the release of UV absorbing substances from a) *S. aureus* ATCC25923, b) *S. aureus* ATCC43300 (MRSA)

- **Compounds 2, 3** and **6** showed the highest growth-inhibitory effect against *S. aureus* and *MRSA*, comparable to **Monolaurin**. **Compound 1** was comparable to Lauric acid. Compound **4** (**β ether**) was less inhibitory than the corresponding ester (compound **3**) and ether analog (compound **2**).
- Antimicrobial activity for **compound 5** was better than the **Caprylic acid** and comparable with **Monocaprylin**.
- The concentration of UV absorbing substances released increased with exposure time for all compounds, with the exception of the laurate ether of methyl β-D-glucopyranoside.

CONCLUSIONS

- It was observed that ether derivatives (compounds **2** and **4**) retained antimicrobial activity, indicating that hydrolysis of the ester bond may not be necessary for antimicrobial activity.
- Alternatively, the role of the carbohydrate moiety (α vs. β) might also play a role, as both ether analogs had different antimicrobial efficacy.
- Membrane damage may account for one part of the mode of action of these compounds as shown by the release of UV absorbing substances.
- The exact mode of action of the different CFA compounds still requires further evaluation.
- The results of the novel CFA derivatives will have clinical significance owing to their activities against MRSA.

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[ii] a) Conley and Kabara, 1973. Antimicrob. Agents Chemother. 4, 501-506
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d) Kitahara, et al. 2006. Int. J. Antimicrob. Agents 27, 51–57
e) Preuss, et al. 2005. Molecular and Cellular Biochem. 272, 29–34
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ACKNOWLEDGMENTS

Funding for this project was provided by TSR Strand I Grant, Irish Government.

Abstract:

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

P. Nobmann, J. Dunne, G. Henehan, P. Bourke

School of Food Science and Env. Health, Dublin Institute of Technology, Dublin, Ireland

Background: MRSA is a global concern. Consequently, new antimicrobial agents are needed. Fatty acids and derivatives have several non-specific modes of action and development of resistance has not been reported[1]. Previous work reported carbohydrate fatty acid (CFA) derivatives with antimicrobial activity against *Listeria* spp. and spoilage bacteria[2].

Objectives: To compare the *in vitro* antimicrobial activity of pure, novel CFA esters and fatty ethers as antimicrobials against *S. aureus* (MRSA). To ascertain the role of free fatty acid and carbohydrate moieties in antimicrobial efficacy, enabling insights into structure/activity relationships and mechanism of action.

Methods: Broth microdilution method to determine minimum inhibitory concentrations (MIC) and effects on the lag phase. Leakage of 260 nm-absorbing materials was examined.

Results: Laurate ether of methyl α-D-glucopyranoside and laurate ester of methyl β-D-glucopyranoside had the highest growth inhibitory effect. Considerable extension of the lag time was observed at sub-MIC values for some compounds. The concentration of UV absorbing material released increased with exposure time for all compounds, except for the laurate ether of methyl β-D-glucopyranoside.

Conclusions: *S. aureus* (MRSA) were significantly inhibited by the derivatives. Membrane damage may account for one part of the mode of action of these compounds. Release of fatty acid may not be essential for antimicrobial activity. Some of the compounds may have clinical significance owing to their activities against MRSA.

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1. Kabara&Marshall. 2005 Medium-chain fatty acids and esters. In *Antimicrobials in foods*. Davidson, Sofos & Brannen, 3rd ed. p. 327-360, CRC Press, Boca Raton, FL

2. Nobmann, P.; Smith, A.; Dunne, J. Henehan, G.; & Bourke, P. 2009 The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. *Int J Food Microbiol* 128 (3), 440-445.

Food Micro conference 2008,
Aberdeen-Scotland, 1st – 4th September 2008.

Synthesis and antimicrobial activity of novel carbohydrate fatty acid derivatives against food pathogens and spoilage microorganisms

Rebecca Hemmings, Aoife Smith, Julie Doyle, Gary Hanahan and Paula Bourke

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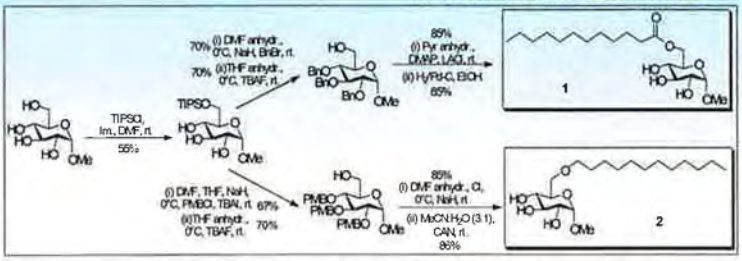
INTRODUCTION

- There is current demand within the food industry for alternative approaches for control of microbial spoilage and safety issues.
- The antimicrobial activity of fatty acids and derivatives has been well documented [i]. Commercially, the most common of these are glycerol monolaurate (ML) and sucrose esters.
- It has been reported[ii] that the half life of monolaurin in cultures of *S. aureus* was *ca.* 5 min, attributed to its cleavage by cellular esterases, which raised the question as to whether the ester conjugate or **free fatty acid** derived from hydrolysis of the ester, was responsible for the antimicrobial activity.
- In contrast to **fatty acid ester conjugates,** corresponding **ether conjugates** should then remain antimicrobial for a longer period of time[iii], assuming the fatty acid component does not require release by esterases for efficacy.

AIMS

The objective of this study was to compare the *in vitro* antimicrobial activity of a range of **pure novel carbohydrate fatty acid derivatives** to ascertain any role of the free fatty acid and/or carbohydrate in antimicrobial activity. **Fatty acid esters** were compared with their corresponding **fatty acid ethers** to investigate the role of the glycoconjugate linkage. The anomeric configuration of the carbohydrate moiety was also investigated to help elucidate any role of the carbohydrate in the antimicrobial efficacy. Free fatty acids (FA) and monoglycerides (MG) were also assessed as controls.

SYNTHESIS & METHODOLOGY



General routes to mono-substituted carbohydrate esters and ethers[iv].

Minimum Inhibitory Concentrations were measured using an absorbance based broth microdilution method to compare compound efficacy.

RESULTS AND DISCUSSION

Microorganism	FA		MG		Carbohydrate fatty acid derivatives					
	LA	CA	ML	MC	1	2	3	4	5	6
L. innocua NCTC 11288	0.63	5	0.04	2.5	0.08	0.04	0.08	5	0.63	0.04
L. monocytogenes ATCC 7644	0.63	> 5	0.04	5	0.08	0.04	0.08	2.5	2.5	0.04
L. monocytogenes NCTC 11994	1.25	> 5	0.04	2.5	0.31	0.04	0.16	> 2.5	1.25	0.04
L. monocytogenes NCTC 7973	1.25	5	0.04	2.5	0.08	0.04	0.16	> 2.5	0.31	0.04
S. aureus ATCC 25923	0.63	5	0.04	2.5	0.31	0.04	0.04	2.5	2.5	0.04
S. aureus (MRSA) ATCC 33591	0.63	> 5	0.04	2.5	0.31	0.04	0.04	2.5	5	0.04
E. coli ATCC 25922	> 20	10	20	5	20	20	20	20	12.5	≥20
E. coli NCTC 12900	12.5	10	12.5	5	12.5	10	12.5	10	12.5	N.D
S. Typhimurium ATCC 14028	> 20	> 20	20	> 20	20	> 20	> 20	20	> 20	N.D
E. aerogenes ATCC 13048	> 20	20	20	10	20	> 20	> 20	> 20	> 20	N.D
P. fluorescens	> 20	5	20	5	> 20	> 20	> 20	> 20	5	N.D

For each analysis the **MIC** was recorded as the concentration (mM) that resulted in total inhibition of all replicates at 37°C after 18 hours. N.D: Not determined
1. Methyl 6-O-lauroyl-α-D-glucopyranoside (***ester***); 2. Methyl 6-O-dodecanyl-α-D-glucopyranoside (***ether***); 3. Methyl 6-O-lauroyl-β-D-glucopyranoside (***ester***); 4. Methyl 6-O-dodecanyl-β-D-glucopyranoside (***ether***); 5. Methyl 6-O-octanoyl-α-D-glucopyranoside (***ester***); 6. Methyl 6-O-lauroyl-α -D-mannopyranoside (***ester***)

- Carbohydrate mono-fatty acid esters and ethers based on glucose and mannose (**1-6**) were synthesised.
- Antimicrobial activity of **compounds 2, 3 and 6** was comparable with Monolaurin against *S. aureus* and *Listeria spp.* **Compound 1** was comparable to Lauric acid. Compound **4** (**β ether**) was less inhibitory than the corresponding ester(Compound **3**).
- Antimicrobial activity for **Compound 5** was comparable with **Monocaprylin** and these compounds were more active than lauric derivatives against *E. coli* and *P. fluorescens.* However all showed compounds had comparatively negligible antimicrobial efficacy for Gram negative microorganisms.

CONCLUSIONS

- Carbohydrate fatty derivatives have been synthesised, showing antimicrobial efficacy comparable with commercially available antimicrobials.
- These results indicate that the anomeric configuration of the carbohydrate and the glycoconjugate linkage play a role in the efficacy, and therefore further optimisation may be possible.
- It was interesting to note that ether derivatives (Compounds **2** and **4**) retained antimicrobial activity indicating that hydrolysis of the ester bond may not be necessary for antimicrobial activity. The role of the carbohydrate in antimicrobial activity requires further evaluation.

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[i] a) Conley and Kabara, 1973. Antimicrob. Agents Chemother. 4, 501-506
b) Dufour, et al, 2003. Int. J. Food Microbiol. 85, 249-256
c) Ferrer, et al. 2005. Enzyme and Microbial Technology 36, 391–398
d) Kitahara, et al. 2006. Int. J. Antimicrob. Agents 27, 51–57
e) Preuss, et al. 2005. Molecular and Cellular Biochem. 272, 29–34
[ii] Ruzin and Novik, 2000. J. Bacteriology 182, 2668-2671
[iii] Ved, et al. 1984. The Journal of Biological Chemistry 259, 8115-8121
[iv] Smith et al. 2008. Carbohydrate Research. *In Press*

ACKNOWLEDGMENTS

Funding for this project was provided by TSR Strand I Grant, Irish Government.

Abstract:

Synthesis And Antimicrobial Activity Of Novel Carbohydrate Fatty Acid Derivatives On Food Pathogens And Food Spoilage Microorganisms.

Patricia Nobmann, Aoife Smith, Julie Dunne, Gary Henehan, Paula Bourke

Dublin Institute of Technology, Dublin, Ireland

There is increased interest within the food industry for novel natural anti-microbial preservatives due to consumer demand for food products with fewer synthetic additives, increased safety, quality and shelf-life. Alternative strategies include the use of novel antimicrobials, such as fatty acid derivatives which have proven bacteriostatic and bactericidal properties against a diverse range of bacteria.

A series of mono-substituted carbohydrate fatty acid (CFA) compounds focusing on caprylic and lauric acids have been synthesized. Chemical routes allowing the attachment of the fatty acid as either an ester or corresponding ether to the monosaccharide 6-hydroxyl were developed to synthesize carbohydrate derivatives with structural differences, enabling comparative studies on the structure/activity relationship for antimicrobial efficacy and mechanism of action.

The range of synthesized CFA derivatives were assessed for in vitro antimicrobial effects against different pathogenic and spoilage bacteria and their efficacy was compared with commercially available compounds with proven antimicrobial activity such as monolaurin and monocaprylin, as well as the pure free fatty acids, lauric acid and caprylic acid. An absorbance based broth microdilution assay was used to measure the minimum inhibitory concentration (MIC) and increase in lag phase.

The antimicrobial efficacy of the CFA derivatives was comparable to monolaurin. CFA derivatives were significantly more effective against Gram positive bacteria, with MIC values between 0.04mM and 0.16mM, than Gram negative bacteria with MIC values of $\geq$20 mM.

Among the carbohydrate derivatives synthesized, the laurate ether of methyl α-D-glucopyranoside and laurate ester of methyl β-D-glucopyranoside showed the highest growth inhibitory effect.

The synthesis 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 is involved in the antimicrobial activity. Thus, CFA derivatives with different structures have great potential for developing antibacterial agents relevant to the food industry.

60th Irish Universities Chemistry Research Colloquium,
University College Cork, 11th-13th June 2008.

Synthesis of novel carbohydrate fatty acid derivatives, antimicrobial efficacy and structure/activity relationship

Patricia Nakimbugwe, Aoife Smith, Julie Dunne*, Paula Bourke and Gary Hastedian

School of Food Science and Environmental Health.
Dublin Institute of Technology (DIT). Cathal Brugha St., Dublin 1, Ireland.

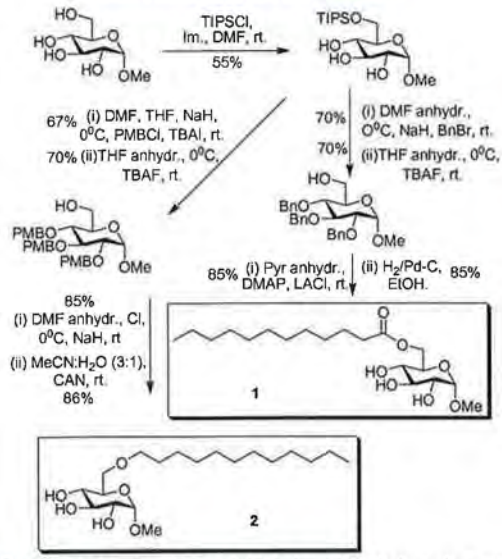
INTRODUCTION

- Alternative approaches for microbial control and shelf life extension include the use of naturally derived antimicrobials such as fatty acid derivatives which have proven bacteriostatic and bactericidal properties against a diverse range of bacteria.
- In addition, recent studies have shown fatty acids, particularly lauric acid, combined with existing antimicrobial agents show activity against methicillin-resistant Staphylococcus aureus (MRSA) and therefore show potential in the healthcare industry.
- This project involves the design and synthesis of a series of carbohydrate fatty acid ester and ether derivatives.

AIMS

- To investigate if the antimicrobial activity of fatty acid compounds can be enhanced using a carbohydrate delivery system.
- To synthesise derivatives with structural differences to enable comparative studies.
- To compare carbohydrate delivery systems with non-biological polyhydroxylated systems.

SYNTHESIS & METHODOLOGY

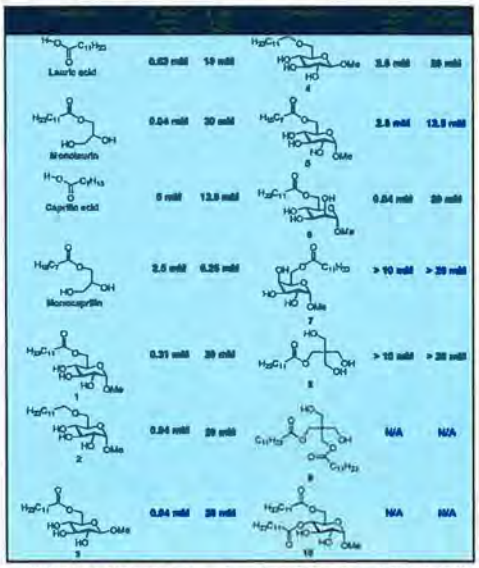


•General routes to mono-substituted carbohydrate esters and ethers.

• Microtiter plate assay

RESULTS AND DISCUSSION

- A series of structurally related carbohydrate mono-fatty acid ester and ether derivatives based on glucose, galactose and mannose were synthesised **(1-7).**
- Non-carbohydrate polyhydroxylated ester derivatives were also synthesised for comparative purposes **(8-9).**
- In addition, a carbohydrate di-fatty acid ester derivative was synthesised **(10)**
- The antimicrobial efficacy reported as Minimum Inhibitory Concentration (MIC), is shown in the table below.



*MIC: value at which no bacterial growth was detected after 18hrs. N.A: Not Available

- The antimicrobial activity of Compounds **2, 3** and **6** were comparable with Monolaurin® against *S. aureus.*
- The derivatives **1, 4** and **7** was less inhibitory
- The antimicrobial activity of Compound **5** was comparable with monocaprylin against *S. aureus*.
- By comparison, none of the synthesised compounds had antimicrobial efficacy against *E. coli*.
- There was no antimicrobial activity associated with the mono substituted pentaerythritol **8**.
- The results for di-substituted derivatives **9** and **10** are not available due to poor solubility in the test medium.

CONCLUSIONS

- Carbohydrate fatty derivatives have been synthesised which have antimicrobial efficacy comparable with commercially available antimicrobials against *S. aureus.*
- These results indicate that the nature of the carbohydrate plays a vital role in the efficacy, and therefore further optimisation may be possible.

ACKNOWLEDGMENTS

- We would like to thank Geraldine Fitzpatrick, Dr Ken Glass and Dr Yannick Ortin, NMR Center UCD, and Dr Dilip Rye, Mass Spectroscopy Center UCD for spectra.
- Funding for this project was provided by TSR Strand I Grant.

Abstract:

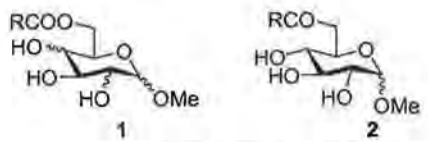
Synthesis, antimicrobial efficacy and structure/activity relationship of novel carbohydrate fatty acid derivatives

Patricia Nobmann, Aoife Smith, Paula Bourke, Julie Dunne, Gary Henehan
Dublin Institute of Technology. Cathal Brugha Street, Dublin 1, Ireland.

The antimicrobial activity of fatty acids and derivatives has been well documented.[1] Commercially, the most common of these are glycerol monolaurate (Lauricidin®) and sucrose esters. There is current demand within the food industry for alternative approaches, including the use of novel antimicrobials such as fatty acid esters, for control of microbial spoilage and safety issues. Recent studies reported that fatty acids, particularly lauric acid, combined with existing antimicrobial agents have activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and that carbohydrate esters can affect the growth of *S. sobrinus*, therefore these compounds have potential in the healthcare industry. [2]

The objectives of this study were to compare the in vitro anti-microbial activity of a range of pure novel carbohydrate fatty acid esters (**1**) with the corresponding fatty acid ethers (**2**) as well as commercial fatty acids and monoglycerides to ascertain any role of the free fatty acid and/or carbohydrate in the antimicrobial efficacy. The antimicrobial efficacy of these compounds was compared quantitatively to allow an estimation of the enhancement of the efficacy over the free fatty acids and commercial standards. Although much work in this area has focused on enzymatic synthesis of sugar derivatives for commercial application, this work has designed synthetic routes to allow the production of pure, novel, regiochemically defined monosaccharide fatty acid esters, and their corresponding ethers. The synthesis of carbohydrate derivatives with structural differences enables comparative studies to attain information on the structure/activity relationship with respect to antimicrobial efficacy and mechanism of action. The contribution of fatty acid chain length to the antimicrobial activity was also investigated. The antimicrobial efficacy of the compounds was measured using an absorbance based broth microdilution assay to determine the minimum inhibitory concentration (MIC).

The synthesis 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 is involved in the antimicrobial activity.



1 **2**

R = $C_{11}H_{23}$ or C_7H_{15}

[1] a) Bergsson, G., *et al*, *International Journal of Antimicrobial Agents*, **2002**, *20*, 258-262.
b) Dufour, M., et al, *International Journal of Food Microbiology*, **2003**, *85*, 249-258.
c) Preuss, H., et al. *Molecular and Cellular Biochemistry*, **2005**, 272, 29–34.

[2] a)Kitahara, T, *et al*, *International Journal of Antimicrobial Agents*, **2006**, *27*, 51-57.
b)Devulapalle, K.S., *et al*. Carbohydrate Research **2004**, 339, 1029-1034.

59th Irish Universities Chemistry Research Colloquium
Dublin City University, 14th-15th June 2007

Synthesis and antimicrobial evaluation of novel carbohydrate fatty acid derivatives

Aoife Smith, Patricia Nobmann, Julie Dunne, Paula Bourke and Gary Henehan.

School of Food Science and Environmental Health.
Dublin Institute of Technology (DIT), Cathal Brugha St., Dublin 1, Ireland.

INTRODUCTION

- There is increased interest in the food industry for antimicrobial preservatives that are perceived as more "natural".
- Alternative strategies for microbial control and shelf life extension include the use of novel antimicrobials.
- Example: fatty acid derivatives which have proven bacteriostatic and bactericidal properties against a diverse range of bacteria.

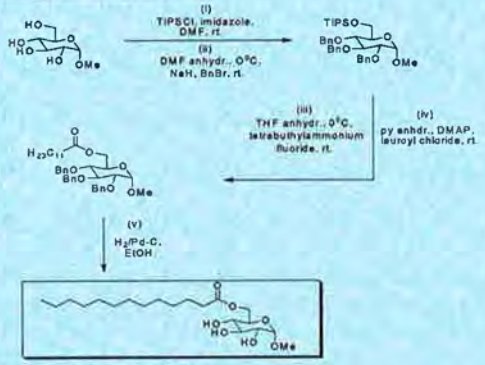
AIMS

- This project involves the design and synthesis of novel mono-substituted carbohydrate fatty acid ester and ether derivatives.
- Investigates whether the antimicrobial activity of fatty acid compounds can be enhanced using a carbohydrate delivery system.

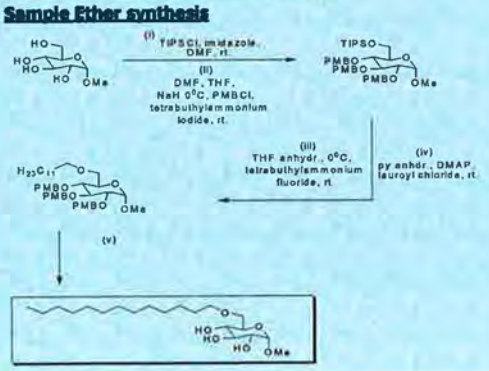
SYNTHESIS, STRUCTURES & METHODOLOGY

- A series of protection/deprotection steps whereby the fatty acid is attached to the 6- OH position.
- The synthesis of carbohydrate derivatives with structural differences enables comparative studies.
- Antimicrobial efficacy tested with the microbroth dilution method.

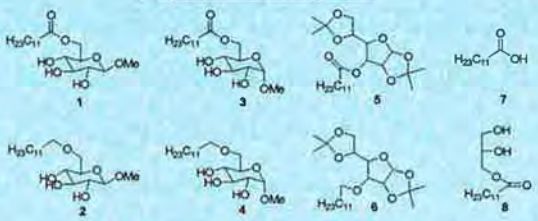
Sample Ester synthesis



Sample Ether synthesis



Synthesised Compounds



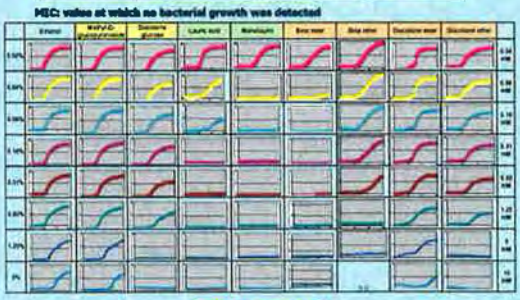
RESULTS AND DISCUSSION

- Six compounds, their corresponding sugars, and lauric acid and monolaurin as controls were tested against two Gram + (*Listeria innocua* and *Staphylococcus aureus*) and one Gram - (*Escherichia coli*) microorganisms.
- Esters comparable with monolaurin against Gram +
- None of the compounds had comparable activity against *Gram* -
- Ether compounds were less inhibitory than the ester forms.
- Protected compounds had no effect on any of the microorganisms tested.

Minimal inhibitory concentration (MIC) values

Compound	*L. innocua*	*S. aureus*	*E. coli*
1	0.08 mM	0.08 mM	> 20 mM
2			
3	0.08 mM	0.08 mM	> 20 mM
4	5 mM	2.5 mM	N.D
5	> 10 mM	> 10 mM	> 20 mM
6	5 mM	> 10 mM	> 20 mM
7	0.31 mM	0.31 mM	> 20 mM
8	0.08 mM	0.08 mM	> 20 mM

MIC: value at which no bacterial growth was detected



CONCLUSIONS

The results obtained in this study showed an antimicrobial effect of the Carbohydrate Fatty Acid derivatives synthesised against Gram positive bacteria.

ACKNOWLEDGMENTS

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Abstract:

Synthesis of novel carbohydrate fatty acid derivatives and the relationship of structure with anti-microbial efficacy

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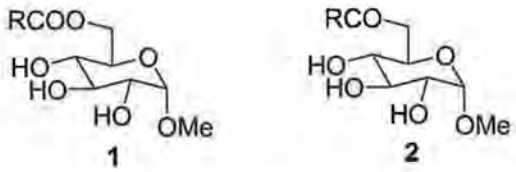
There is increased interest within the food industry for anti-microbial preservatives that are perceived as more "natural" due to consumer demand for food products with fewer synthetic additives but increased safety, quality and shelf-life. . Alternative strategies for microbial control and shelf life extension include the use of novel antimicrobials, such as fatty acid derivatives which have proven bacteriostatic and bactericidal properties against a diverse range of bacteria.[1,2]

Among such fatty acid derivatives are carbohydrate fatty acid esters, which have previously been generated enzymatically to yield non-regiocontrolled mixtures. The current work employs a protection / deprotection strategy allowing the divergent synthesis of pure compounds with a fatty acid attached to a carbohydrate primary hydroxyl. Altering the carbohydrate and the fatty acid enables comparative studies to attain information on the structure / activity relationship with anti-microbial efficacy and mechanism of action. To date, a series of glucopyranoside fatty acid esters (**1**) and ethers (**2**) focusing on caprylic and lauric acids have been synthesised. The antimicrobial efficacy of these novel compounds compared with commercially available standards such as monolaurin and pure fatty acids is under evaluation against common food pathogen organisms. The synthesis of these compounds, along with preliminary microbiological results, will be presented. This group of compounds has numerous potential applications within the food and healthcare industries.



1 **2**

$R = C_{11}H_{23}$

[1] Kitahara, T. *International Journal of Antimicrobial Agents*, **2006**, *27*, 51-57.
[1] Glass, K. A.; Johnson, E. A. *Food Microbiology*, **2004**, *21*, 675-682.

APPENDIX 2

BIOLOG **Phenotype MicroArrays™**

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 M-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 M-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 i-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 I-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 α-Keto Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanone	H12 3-Hydroxy 2-Butanone

BIOLOG **Phenotype MicroArrays™**

PM3B MicroPlate™ Nitrogen Sources

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D-1 N-Acetyl-D,L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyroglutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamin e	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 β-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alloxan	G5 Allantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 α-Amino-N-Butyric Acid	G9 ε-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 δ-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

PM4A MicroPlate™ Phosphorus and Sulfur Sources

A1 Negative Control	A2 Phosphate	A3 Pyrophosphate	A4 Trimeta-phosphate	A5 Tripoly-phosphate	A6 Triethyl Phosphate	A7 Hypophosphite	A8 Adenosine- 2'- monophosphate	A9 Adenosine- 3'- monophosphate	A10 Adenosine- 5'- monophosphate	A11 Adenosine- 2',3'-cyclic monophosphate	A12 Adenosine- 5',5'-cyclic monophosphate
B1 Thiophosphate	B2 Dithiophosphat e	B3 D,L-α-Glycerol Phosphate	B4 β-Glycerol Phosphate	B5 Carbamyl Phosphate	B6 D-2-Phospho-Glyceric Acid	B7 D-3-Phospho-Glyceric Acid	B8 Guanosine- 2'- monophosphate	B9 Guanosine- 3'- monophosphate	B10 Guanosine- 5'- monophosphate	B11 Guanosine- 2',3'-cyclic monophosphate	B12 Guanosine- 5',5'-cyclic monophosphate
C1 Phosphonoacetic Acid	C2 Phospho-Glycolic Acid	C3 D-Glucose-1-Phosphate	C4 D-Glucose-6-Phosphate	C5 2-Deoxy-D-Glucose 6-Phosphate	C6 D-Glucosamine-6-Phosphate	C7 6-Phospho-Gluconic Acid	C8 Cytidine- 2'- monophosphate	C9 Cytidine- 3'- monophosphate	C10 Cytidine- 5'- monophosphate	C11 Cytidine- 2',3'-cyclic monophosphate	C12 Cytidine- 5',6'-cyclic monophosphate
D1 D-Mannose-1-Phosphate	D2 D-Mannose-6-Phosphate	D3 Cysteamine-S-Phosphate	D4 Phospho-L-Arginine	D5 O-Phospho-D-Serine	D6 O-Phospho-L-Serine	D7 O-Phospho-L-Threonine	D8 Uridine- 2'- monophosphate	D9 Uridine- 3'- monophosphate	D10 Uridine- 5'- monophosphate	D11 Uridine- 2',3'-cyclic monophosphate	D12 Uridine- 3',5'-cyclic monophosphate
E1 O-Phospho-D-Tyrosine	E2 O-Phospho-L-Tyrosine	E3 Phosphocreatin e	E4 Phosphoryl Choline	E5 O-Phosphoryl-Ethanolamine	E6 Phosphono Acetic Acid	E7 2-Aminoethyl Phosphonic Acid	E8 Methylene Diphosphonic Acid	E9 Thymidine- 3'- monophosphate	E10 Thymidine- 5'- monophosphate	E11 Inositol Hexaphosphate	E12 Thymidine 3',5'- cyclic monophosphate
F1 Negative Control	F2 Sulfate	F3 Thiosulfate	F4 Tetrathionate	F5 Thiophosphate	F6 Dithiophosphat e	F7 L-Cysteine	F8 D-Cysteine	F9 L-Cysteinyl-Glycine	F10 L-Cystein Acid	F11 Cysteamine	F12 L-Cysteine Sulfonic Acid
G1 N-Acetyl-L-Cysteine	G2 S-Methyl-L-Cysteine	G3 Cystathionine	G4 Lanthionine	G5 Djenkolic Acid	G6 D,L-Ethionine	G7 L-Methionine	G8 D-Methionine	G9 Glycyl-L-Methionine	G10 N-Acetyl-D,L-Methionine	G11 L- Methionine Sulfoxide	G12 L-Methionine Sulfone
H1 L-Djenkolic Acid	H2 Thiourea	H3 1-Thio-β-D-Glucose	H4 D,L-Lipoamide	H5 Taurocholic Acid	H6 Taurine	H7 Hypotaurine	H8 e-Amino Butyric Sulfonic Acid	H9 Butane Sulfonic Acid	H10 2-Hydroxyethane Sulfonic Acid	H11 Methane Sulfonic Acid	H12 Tetramethylene Sulfone

BIOLOG

Phenotype MicroArrays™

PM5 MicroPlate™ Nutrient Supplements

A1 Negative Control	A2 Positive Control	A3 L-Alanine	A4 L-Arginine	A5 L-Asparagine	A6 L-Aspartic Acid	A7 L-Cysteine	A8 L-Glutamic Acid	A9 Adenosine-3',5'-cyclic monophosphate	A10 Adenine	A11 Adenosine	A12 2'-Deoxy Adenosine
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 Guanosine-3',5'-cyclic monophosphate	B10 Guanine	B11 Guanosine	B12 2'-Deoxy Guanosine
C1 L-Proline	C2 L-Serine	C3 L-Threonine	C4 L-Tryptophan	C5 L-Tyrosine	C6 L-Valine	C7 L-Isoleucine + L-Valine	C8 trans-4-Hydroxy L-Proline	C9 (S) 4-Amino-imidazole-4(5)-Carboxamide	C10 Hypoxanthine	C11 Inosine	C12 2'-Deoxy Inosine
D1 L-Ornithine	D2 L-Citrulline	D3 Chorismic Acid	D4 (-)-Shikimic Acid	D5 L-Homoserine Lactone	D6 D-Alanine	D7 D-Aspartic Acid	D8 D-Glutamic Acid	D9 D,L-α,ε-Diamino-pimelic Acid	D10 Cytosine	D11 Cytidine	D12 2'-Deoxy Cytidine
E1 Putrescine	E2 Spermidine	E3 Spermine	E4 Pyridoxine	E5 Pyridoxal	E6 Pyridoxamine	E7 β-Alanine	E8 D-Pantothenic Acid	E9 Orotic Acid	E10 Uracil	E11 Uridine	E12 2'-Deoxy Uridine
F1 Quinolinic Acid	F2 Nicotinic Acid	F3 Nicotinamide	F4 β-Nicotinamide Adenine Dinucleotide	F5 δ-Amino-Levulinic Acid	F6 Hematin	F7 Deferoxamine Mesylate	F8 D-(+)-Glucose	F9 N-Acetyl D-Glucosamine	F10 Thymine	F11 Glutathione (reduced form)	F12 Thymidine
G1 Oxaloacetic Acid	G2 D-Biotin	G3 Cyano-Cobalamine	G4 p-Amino-Benzoic Acid	G5 Folic Acid	G6 Inosine + Thiamine	G7 Thiamine	G8 Thiamine Pyrophosphate	G9 Riboflavin	G10 Pyrroxine Quinoline Quinone	G11 Menadione	G12 Myo-Inositol
H1 Butyric Acid	H2 D,L-α-Hydroxy-Butyric Acid	H3 α-Ketobutyric Acid	H4 Caprylic Acid	H5 D,L-α-Lipoic Acid (oxidized form)	H6 D,L-Mevalonic Acid	H7 D,L-Carnitine	H8 Choline	H9 Tween 20	H10 Tween 40	H11 Tween 60	H12 Tween 80

PM6 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Ala-Asn	A4 Ala-Arg	A5 Ala-Asn	A6 Ala-Glu	A7 Ala-Gly	A8 Ala-His	A9 Ala-Leu	A10 Ala-Lys	A11 Ala-Phe	A12 Ala-Pro
B1 Ala-Ser	B2 Ala-Thr	B3 Ala-Trp	B4 Ala-Tyr	B5 Arg-Ala	B6 Arg-Arg	B7 Arg-Asp	B8 Arg-Gln	B9 Arg-Glu	B10 Arg-He	B11 Arg-Leu	B12 Arg-Lys
C1 Arg-Met	C2 Arg-Phe	C3 Arg-Ser	C4 Arg-Trp	C5 Arg-Tyr	C6 Arg-Val	C7 Asn-Glu	C8 Asn-Val	C9 Asp-Asp	C10 Asp-Glu	C11 Asp-Leu	C12 Asp-Lys
D1 Asp-Phe	D2 Asp-Trp	D3 Asp-Val	D4 Cys-Gly	D5 Glu-Glu	D6 Gln-Gly	D7 Glu-Asp	D8 Glu-Gln	D9 Glu-Gly	D10 Glu-Ser	D11 Glu-Trp	D12 Glu-Tyr
E1 Glu-Val	E2 Gly-Ala	E3 Gly-Arg	E4 Gly-Cys	E5 Gly-Gly	E6 Gly-His	E7 Gly-Leu	E8 Gly-Lys	E9 Gly-Met	E10 Gly-Phe	E11 Gly-Pro	E12 Gly-Ser
F1 Gly-Thr	F2 Gly-Trp	F3 Gly-Tyr	F4 Gly-Val	F5 His-Asp	F6 His-Gly	F7 His-Leu	F8 His-Lys	F9 His-Met	F10 His-Pro	F11 His-Ser	F12 His-Trp
G1 His-Tyr	G2 His-Val	G3 Ile-Ala	G4 Ile-Arg	G5 Ile-Gln	G6 Ile-Gly	G7 Ile-His	G8 Ile-Ile	G9 Ile-Met	G10 Ile-Phe	G11 Ile-Pro	G12 Ile-Ser
H1 Ile-Trp	H2 Ile-Tyr	H3 Ile-Val	H4 Leu-Ala	H5 Leu-Arg	H6 Leu-Asp	H7 Leu-Gln	H8 Leu-Gly	H9 Leu-Ile	H10 Leu-Leu	H11 Leu-Met	H12 Leu-Phe

BIOLOG

Phenotype MicroArrays™

PM7 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Leu-Ser	A4 Leu-Trp	A5 Leu-Val	A6 Lys-Ala	A7 Lys-Arg	A8 Lys-Glu	A9 Lys-Ile	A10 Lys-Leu	A11 Lys-Lys	A12 Lys-Phe
B1 Lys-Pro	B2 Lys-Ser	B3 Lys-Thr	B4 Lys-Trp	B5 Lys-Tyr	B6 Lys-Val	B7 Met-Arg	B8 Met-Asp	B9 Met-Gln	B10 Met-Glu	B11 Met-Gly	B12 Met-His
C1 Met-Ile	C2 Met-Leu	C3 Met-Lys	C4 Met-Met	C5 Met-Phe	C6 Met-Pro	C7 Met-Trp	C8 Met-Val	C9 Phe-Ala	C10 Phe-Gly	C11 Phe-Ile	C12 Phe-Phe
D1 Phe-Pro	D2 Phe-Ser	D3 Phe-Trp	D4 Pro-Ala	D5 Pro-Asp	D6 Pro-Gln	D7 Pro-Gly	D8 Pro-Hyp	D9 Pro-Leu	D10 Pro-Phe	D11 Pro-Pro	D12 Pro-Tyr
E1 Ser-Ala	E2 Ser-Gly	E3 Ser-His	E4 Ser-Leu	E5 Ser-Met	E6 Ser-Phe	E7 Ser-Pro	E8 Ser-Ser	E9 Ser-Tyr	E10 Ser-Val	E11 Thr-Ala	E12 Thr-Arg
F1 Thr-Glu	F2 Thr-Gly	F3 Thr-Leu	F4 Thr-Met	F5 Thr-Pro	F6 Trp-Ala	F7 Trp-Arg	F8 Trp-Asp	F9 Trp-Glu	F10 Trp-Gly	F11 Trp-Leu	F12 Trp-Lys
G1 Trp-Phe	G2 Trp-Ser	G3 Trp-Trp	G4 Trp-Tyr	G5 Tyr-Ala	G6 Tyr-Gln	G7 Tyr-Glu	G8 Tyr-Gly	G9 Tyr-His	G10 Tyr-Leu	G11 Tyr-Lys	G12 Tyr-Phe
H1 Tyr-Trp	H2 Tyr-Tyr	H3 Val-Arg	H4 Val-Asn	H5 Val-Asp	H6 Val-Gly	H7 Val-His	H8 Val-Ile	H9 Val-Leu	H10 Val-Tyr	H11 Val-Val	H12 Y-Glu-Gly

PM8 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Ala-Asp	A4 Ala-Gln	A5 Ala-Ile	A6 Ala-Met	A7 Ala-Val	A8 Asp-Ala	A9 Asp-Glu	A10 Asp-Gly	A11 Gln-Ala	A12 Gly-Asn
B1 Gly-Asp	B2 Gly-Ile	B3 His-Ala	B4 His-Gln	B5 His-His	B6 Ile-Asn	B7 Ile-Leu	B8 Leu-Asn	B9 Leu-His	B10 Leu-Pro	B11 Leu-Tyr	B12 Lys-Asp
C1 Lys-Gly	C2 Lys-Met	C3 Met-Thr	C4 Met-Tyr	C5 Phe-Asp	C6 Phe-Gln	C7 Gln-Glu	C8 Phe-Met	C9 Phe-Tyr	C10 Phe-Val	C11 Pro-Arg	C12 Pro-Asn
D1 Pro-Gln	D2 Pro-Ile	D3 Pro-Lys	D4 Pro-Ser	D5 Pro-Trp	D6 Pro-Val	D7 Ser-Asn	D8 Ser-Asp	D9 Ser-Gln	D10 Ser-Glu	D11 Thr-Asp	D12 Thr-Gln
E1 Thr-Phe	E2 Thr-Ser	E3 Trp-Val	E4 Tyr-Ile	E5 Tyr-Val	E6 Val-Ala	E7 Val-Gln	E8 Val-Glu	E9 Val-Lys	E10 Val-Met	E11 Val-Phe	E12 Val-Pro
F1 Val-Ser	F2 β-Ala-Ala	F3 β-Ala-Gly	F4 β-Ala-His	F5 Met-β-Ala	F6 β-Ala-Phe	F7 D-Ala-D-Ala	F8 D-Ala-Gly	F9 D-Ala-Leu	F10 D-Leu-D-Leu	F11 D-Leu-Gly	F12 D-Leu-Tyr
G1 Y-Glu-Gly	G2 Y-D-Glu-Gly	G3 Gly-D-Ala	G4 Gly-D-Asp	G5 Gly-D-Ser	G6 Gly-D-Thr	G7 Gly-D-Val	G8 Leu-β-Ala	G9 Leu-D-Leu	G10 Phe-β-Ala	G11 Ala-Ala-Ala	G12 D-Ala-Gly-Gly
H1 Gly-Gly-Ala	H2 Gly-Gly-D-Leu	H3 Gly-Gly-Gly	H4 Gly-Gly-Ile	H5 Gly-Gly-Leu	H6 Gly-Gly-Phe	H7 Val-Tyr-Val	H8 Gly-Phe-Phe	H9 Leu-Gly-Gly	H10 Leu-Leu-Leu	H11 Phe-Gly-Gly	H12 Tyr-Gly-Gly

BIOLOG

Phenotype MicroArrays™

PM9 MicroPlate™ Osmolytes

A1 NaCl 1%	A2 NaCl 2%	A3 NaCl 3%	A4 NaCl 4%	A5 NaCl 5%	A6 NaCl 6.5%	A7 NaCl 8%	A8 NaCl 8.5%	A9 NaCl 7%	A10 NaCl 8%	A11 NaCl 9%	A12 NaCl 10%
B1 NaCl 6%	B2 NaCl 6% + Betaine	B3 NaCl 6% + N-N Dimethyl glycine	B4 NaCl 6% + Sarcosine	B5 NaCl 6% + Dimethyl sulphoxyl propionate	B6 NaCl 6% + MOPS	B7 NaCl 6% + Betaine	B8 NaCl 6% + Choline	B9 NaCl 6% + Phosphoryl choline	B10 NaCl 6% + Creatine	B11 NaCl 6% + Creatinine	B12 NaCl 6% + L- Carnitine
C1 NaCl 6% + KCl	C2 NaCl 6% + L-proline	C3 NaCl 6% + N-Acetyl L-glutamine	C4 NaCl 6% + β-Glutamic acid	C5 NaCl 6% + γ-Amino -α-butyric acid	C6 NaCl 6% + Glutathione	C7 NaCl 6% + Glycerol	C8 NaCl 6% + Trehalose	C9 NaCl 6% + Trimethylamine -N-oxide	C10 NaCl 6% + Trimethylamine	C11 NaCl 6% + Octopine	C12 NaCl 6% + Trigonelline
D-1 Potassium chloride 3%	D2 Potassium chloride 4%	D3 Potassium chloride 5%	D4 Potassium chloride 6%	D5 Sodium sulfate 2%	D6 Sodium sulfate 3%	D7 Sodium sulfate 4%	D8 Sodium sulfate 5%	D9 Ethylene glycol 5%	D10 Ethylene glycol 10%	D11 Ethylene glycol 15%	D12 Ethylene glycol 20%
E1 Sodium formate 1%	E2 Sodium formate 2%	E3 Sodium formate 3%	E4 Sodium formate 4%	E5 Sodium formate 5%	E6 Sodium formate 6%	E7 Urea 2%	E8 Urea 3%	E9 Urea 4%	E10 Urea 5%	E11 Urea 6%	E12 Urea 7%
F1 Sodium Lactate 1%	F2 Sodium Lactate 2%	F3 Sodium Lactate 3%	F4 Sodium Lactate 4%	F5 Sodium Lactate 5%	F6 Sodium Lactate 6%	F7 Sodium Lactate 7%	F8 Sodium Lactate 8%	F9 Sodium Lactate 9%	F10 Sodium Lactate 10%	F11 Sodium Lactate 11%	F12 Sodium Lactate 12%
G1 Sodium Phosphate pH 7 20mM	G2 Sodium Phosphate pH 7 50mM	G3 Sodium Phosphate pH 7 100mM	G4 Sodium Phosphate pH 7 200mM	G5 Sodium Benzoate pH 5.2 20mM	G6 Sodium Benzoate pH 5.2 50mM	G7 Sodium Benzoate pH5.2 100mM	G8 Sodium Benzoate pH 5.2 200mM	G9 Ammonium sulfate pH8 15mM	G10 Ammonium sulfate pH 8 30mM	G11 Ammonium sulfate pH 8 50mM	G12 Ammonium sulfate pH8 100mM
H1 Sodium Nitrate 10mM	H2 Sodium Nitrate 20mM	H3 Sodium Nitrate 40mM	H4 Sodium Nitrate 60mM	H5 Sodium Nitrate 80mM	H6 Sodium Nitrate 100mM	H7 Sodium Nitrite 10mM	H8 Sodium Nitrite 20mM	H9 Sodium Nitrite 40mM	H10 Sodium Nitrite 60mM	H11 Sodium Nitrite 80mM	H12 Sodium Nitrite 100mM

PM10 MicroPlate™ pH

A1 pH 3.0	A2 pH 4	A3 pH 4.3	A4 pH 5	A5 pH 5.5	A6 pH 6	A7 pH 7	A8 pH 8	A9 pH 8.5	A10 pH 9	A11 pH 9.5	A12 pH 10
B1 pH 4.5	B2 pH 4.5 + L-Alanine	B3 pH 4.5 + L-Arginine	B4 pH 4.5 + L-Asparagine	B5 pH 4.5 + L-Aspartic Acid	B6 pH 4.5 + L-Glutamic Acid	B7 pH 4.5 + L-Glutamine	B8 pH 4.5 + Glycine	B9 pH 4.5 + L-Histidine	B10 pH 4.5 + L-Isoleucine	B11 pH 4.5 + L-Leucine	B12 pH 4.5 + L-Lysine
C1 pH 4.5 + L-Methionine	C2 pH 4.5 + L-Phenylalanine	C3 pH 4.5 + L-Proline	C4 pH 4.5 + L-Serine	C5 pH 4.5 + L-Threonine	C6 pH 4.5 + L-Tryptophan	C7 pH 4.5 + L-Tyrosine	C8 pH 4.5 + L-Valine	C9 pH 4.5 + Hydroxy-L-Proline	C10 pH 4.5 + L-Ornithine	C11 pH 4.5 + L-Homarginine	C12 pH 4.5 + L-Homoserine
D-1 pH 4.5 + Anthranilic acid	D2 pH 4.5 + L-Homocystine	D3 pH 4.5 + L-Norvaline	D4 pH 4.5 + α- Amino-N-butyric acid	D5 pH 4.5 + p-Aminobenzoate	D6 pH 4.5 + L-Cysteic acid	D7 pH 4.5 + D-Lysine	D8 pH 4.5 + 5-Hydroxy Lysine	D9 pH 4.5 + 5-Hydroxy Tryptophan	D10 pH 4.5 + D,L-Diaminopimelic acid	D11 pH 4.5 + Trimethyl amine-N-oxide	D12 pH 4.5 + Urea
E1 pH 9.0	E2 pH 9.5 + L-Alanine	E3 pH 9.5 + L-Arginine	E4 pH 9.5 + L-Asparagine	E5 pH 9.5 + L-Aspartic Acid	E6 pH 9.5 + L-Glutamic Acid	E7 pH 9.5 + L-Glutamine	E8 pH 9.5 + Glycine	E9 pH 9.5 + L-Histidine	E10 pH 9.5 + L-Isoleucine	E11 pH 9.5 + L-Leucine	E12 pH 9.5 + L-Lysine
F1 pH 9.5 + L-Methionine	F2 pH 9.5 + L-Phenylalanine	F3 pH 9.5 + L-Proline	F4 pH 9.5 + L-Serine	F5 pH 9.5 + L-Threonine	F6 pH 9.5 + L-Tryptophan	F7 pH 9.5 + L-Tyrosine	F8 pH 9.5 + L-Valine	F9 pH 9.5 + Hydroxy-L-Proline	F10 pH 9.5 + L-Ornithine	F11 pH 9.5 + L-Homarginine	F12 pH 9.5 + L-Homoserine
G1 pH 9.5 + Anthranilic acid	G2 pH 9.5 + L-Homocystine	G3 pH 9.5 + L-Norvaline	G4 pH 9.5 + Agmatine	G5 pH 9.5 + Cadaverine	G6 pH 9.5 + Putrescine	G7 pH 9.5 + Histamine	G8 pH 9.5 + Phenylethylamine	G9 pH 9.5 + Tyramine	G10 pH 9.5 + Creatine	G11 pH 9.5 + Trimethyl amine-N-oxide	G12 pH 9.5 + Urea
H1 N-Caprylate	H2 N-α-D-Glucoside	H3 N-β-D-Glucoside	H4 N-α-D-Galactoside	H5 N-β-D-Galactoside	H6 N-α- D-Glucuronide	H7 N-β- D-Glucuronide	H8 N-β-D-Glucosaminide	H9 N-β-D-Galactosaminid e	H10 N-α-D-Mannoside	H11 N-PO4	H12 N-BO4

PM11C MicroPlate™

A1 Amikacin 1	A2 Amikacin 2	A3 Amikacin 3	A4 Amikacin 4	A5 Chlortetracycline 1	A6 Chlortetracycline 2	A7 Chlortetracycline 3	A8 Chlortetracycline 4	A9 Lincomycin 1	A10 Lincomycin 2	A11 Lincomycin 3	A12 Lincomycin 4
B1 Amoxicillin 1	B2 Amoxicillin 2	B3 Amoxicillin 3	B4 Amoxicillin 4	B5 Cloxacillin 1	B6 Cloxacillin 2	B7 Cloxacillin 3	B8 Cloxacillin 4	B9 Lomefloxacin 1	B10 Lomefloxacin 2	B11 Lomefloxacin 3	B12 Lomefloxacin 4
C1 Bleomycin 1	C2 Bleomycin 2	C3 Bleomycin 3	C4 Bleomycin 4	C5 Colistin 1	C6 Colistin 2	C7 Colistin 3	C8 Colistin 4	C9 Minocycline 1	C10 Minocycline 2	C11 Minocycline 3	C12 Minocycline 4
D1 Capreomycin 1	D2 Capreomycin 2	D3 Capreomycin 3	D4 Capreomycin 4	D5 Demeclocycline 1	D6 Demeclocycline 2	D7 Demeclocycline 3	D8 Demeclocycline 4	D9 Nafcillin 1	D10 Nafcillin 2	D11 Nafcillin 3	D12 Nafcillin 4
E1 Cefazolin 1	E2 Cefazolin 2	E3 Cefalotin 3	E4 Cefalotin 4	E5 Enoxacin 1	E6 Enoxacin 2	E7 Enoxacin 3	E8 Enoxacin 4	E9 Nalidixic acid 1	E10 Nalidixic acid 2	E11 Nalidixic acid 3	E12 Nalidixic acid 4
F1 Chloramphenicol 1	F2 Chloramphenicol 2	F3 Chloramphenicol 3	F4 Chloramphenicol 4	F5 Erythromycin 1	F6 Erythromycin 2	F7 Erythromycin 3	F8 Erythromycin 4	F9 Neomycin 1	F10 Neomycin 2	F11 Neomycin 3	F12 Neomycin 4
G1 Ceftriaxone 1	G2 Ceftriaxone 2	G3 Ceftriaxone 3	G4 Ceftriaxone 4	G5 Gentamicin 1	G6 Gentamicin 2	G7 Gentamicin 3	G8 Gentamicin 4	G9 Potassium tellurite 1	G10 Potassium tellurite 2	G11 Potassium tellurite 3	G12 Potassium tellurite 4
H1 Cephalothin 1	H2 Cephalothin 2	H3 Cephalothin 3	H4 Cephalothin 4	H5 Kanamycin 1	H6 Kanamycin 2	H7 Kanamycin 3	H8 Kanamycin 4	H9 Ofloxacin 1	H10 Ofloxacin 2	H11 Ofloxacin 3	H12 Ofloxacin 4

PM12B MicroPlate™

A1 Penicillin G 1	A2 Penicillin G 2	A3 Penicillin G 3	A4 Penicillin G 4	A5 Tetracycline 1	A6 Tetracycline 2	A7 Tetracycline 3	A8 Tetracycline 4	A9 Carbenicillin 1	A10 Carbenicillin 2	A11 Carbenicillin 3	A12 Carbenicillin 4
B1 Oxacillin 1	B2 Oxacillin 2	B3 Oxacillin 3	B4 Oxacillin 4	B5 Perimepicycline 1	B6 Perimepicycline 2	B7 Perimepicycline 3	B8 Perimepicycline 4	B9 Polymyxin B 1	B10 Polymyxin B 2	B11 Polymyxin B 3	B12 Polymyxin B 4
C1 Paromomycin 1	C2 Paromomycin 2	C3 Paromomycin 3	C4 Paromomycin 4	C5 Vancomycin 1	C6 Vancomycin 2	C7 Vancomycin 3	C8 Vancomycin 4	C9 D,L-Serine hydroxamate 1	C10 D,L-Serine hydroxamate 2	C11 D,L-Serine hydroxamate 3	C12 D,L-Serine hydroxamate 4
D1 Streptomycin 1	D2 Streptomycin 2	D3 Streptomycin 3	D4 Streptomycin 4	D5 Sulfamethazine 1	D6 Sulfamethazine 2	D7 Sulfamethazine 3	D8 Sulfamethazine 4	D9 Novobiocin 1	D10 Novobiocin 2	D11 Novobiocin 3	D12 Novobiocin 4
E1 2,4-Diamino-6,7-diisopropyl-pteridine 1	E2 2,4-Diamino-6,7-diisopropyl-pteridine 2	E3 2,4-Diamino-6,7-diisopropyl-pteridine 3	E4 2,4-Diamino-6,7-diisopropyl-pteridine 4	E5 Sulfadiazine 1	E6 Sulfadiazine 2	E7 Sulfadiazine 3	E8 Sulfadiazine 4	E9 Benzethonium chloride 1	E10 Benzethonium chloride 2	E11 Benzethonium chloride 3	E12 Benzethonium chloride 4
F1 Tobramycin 1	F2 Tobramycin 2	F3 Tobramycin 3	F4 Tobramycin 4	F5 Sulfadiazole 1	F6 Sulfadiazole 2	F7 Sulfadiazole 3	F8 Sulfathiazole 4	F9 5-Fluorootic acid 1	F10 5-Fluorootic acid 2	F11 5-Fluorootic acid 3	F12 5-Fluorootic acid 4
G1 Spectinomycin 1	G2 Spectinomycin 2	G3 Spectinomycin 3	G4 Spectinomycin 4	G5 Sulfa-methoxazole 1	G6 Sulfa-methoxazole 2	G7 Sulfa-methoxazole 3	G8 Sulfa-methoxazole 4	G9 L-Aspartic-β-hydroxamate 1	G10 L-Aspartic-β-hydroxamate 2	G11 L-Aspartic-β-hydroxamate 3	G12 L-Aspartic-β-hydroxamate 4
H1 Spiramycin 1	H2 Spiramycin 2	H3 Spiramycin 3	H4 Spiramycin 4	H5 Rifampicin 1	H6 Rifampicin 2	H7 Rifampicin 3	H8 Rifampicin 4	H9 Dodecyltrimethyl ammonium bromide 1	H10 Dodecyltrimethyl ammonium bromide 2	H11 Dodecyltrimethyl ammonium bromide 3	H12 Dodecyltrimethyl ammonium bromide 4

BIOLOG Phenotype MicroArrays™

PM13B MicroPlate™

A1 Ampicillin 1	A2 Ampicillin 2	A3 Ampicillin 3	A4 Ampicillin 4	A5 Dequalinium chloride 1	A6 Dequalinium chloride 2	A7 Dequalinium chloride 3	A8 Dequalinium chloride 4	A9 Nickel chloride 1	A10 Nickel chloride 2	A11 Nickel chloride 3	A12 Nickel chloride 4
B1 Azlocillin 1	B2 Azlocillin 2	B3 Azlocillin 3	B4 Azlocillin 4	B5 2, 2'-Dipyridyl 1	B6 2, 2'-Dipyridyl 2	B7 2, 2'-Dipyridyl 3	B8 2, 2'-Dipyridyl 4	B9 Oxolinic acid 1	B10 Oxolinic acid 2	B11 Oxolinic acid 3	B12 Oxolinic acid 4
C1 6-Mercapto-purine 1	C2 6-Mercapto-purine 2	C3 6-Mercapto-purine 3	C4 6-Mercapto-purine 4	C5 Doxycycline 1	C6 Doxycycline 2	C7 Doxycycline 3	C8 Doxycycline 4	C9 Potassium chromate 1	C10 Potassium chromate 2	C11 Potassium chromate 3	C12 Potassium chromate 4
D1 Cefuroxime 1	D2 Cefuroxime 2	D3 Cefuroxime 3	D4 Cefuroxime 4	D5 5-Fluorouracil 1	D6 5-Fluorouracil 2	D7 5-Fluorouracil 3	D8 5-Fluorouracil 4	D9 Rolitetracycline 1	D10 Rolitetracycline 2	D11 Rolitetracycline 3	D12 Rolitetracycline 4
E1 Cytosine-1-beta-D-arabino-furanoside 1	E2 Cytosine-1-beta-D-arabino-furanoside 2	E3 Cytosine-1-beta-D-arabino-furanoside 3	E4 Cytosine-1-beta-D-arabino-furanoside 4	E5 Geneticin (G418) 1	E6 Geneticin (G418) 2	E7 Geneticin (G418) 3	E8 Geneticin (G418) 4	E9 Rothenium red 1	E10 Rothenium red 2	E11 Rothenium red 3	E12 Rothenium red 4
F1 Cesium chloride 1	F2 Cesium chloride 2	F3 Cesium chloride 3	F4 Cesium chloride 4	F5 Glycine 1	F6 Glycine 2	F7 Glycine 3	F8 Glycine 4	F9 Thallium (I) acetate 1	F10 Thallium (I) acetate 2	F11 Thallium (I) acetate 3	F12 Thallium (I) acetate 4
G1 Cobalt chloride 1	G2 Cobalt chloride 2	G3 Cobalt chloride 3	G4 Cobalt chloride 4	G5 Manganese chloride 1	G6 Manganese chloride 2	G7 Manganese chloride 3	G8 Manganese chloride 4	G9 Trifluoperazine 1	G10 Trifluoperazine 2	G11 Trifluoperazine 3	G12 Trifluoperazine 4
H1 Cupric chloride 1	H2 Cupric chloride 2	H3 Cupric chloride 3	H4 Cupric chloride 4	H5 Mecasermin 1	H6 Mecasermin 2	H7 Mecasermin 3	H8 Mecasermin 4	H9 Tylosin 1	H10 Tylosin 2	H11 Tylosin 3	H12 Tylosin 4

PM14A MicroPlate™

A1 Acriflavine 1	A2 Acriflavine 2	A3 Acriflavine 3	A4 Acriflavine 4	A5 Puromycin 1	A6 Puromycin 2	A7 Puromycin 3	A8 Puromycin 4	A9 Sanguinarine 1	A10 Sanguinarine 2	A11 Sanguinarine 3	A12 Sanguinarine 4
B1 9-Aminoacridine 1	B2 9-Aminoacridine 2	B3 9-Aminoacridine 3	B4 9-Aminoacridine 4	B5 Fusaric acid 1	B6 Fusaric acid 2	B7 Fusaric acid 3	B8 Fusaric acid 4	B9 Sodium arsenate 1	B10 Sodium arsenate 2	B11 Sodium arsenate 3	B12 Sodium arsenate 4
C1 Boric Acid 1	C2 Boric Acid 2	C3 Boric Acid 3	C4 Boric Acid 4	C5 1-Hydroxy-pyridine -2-thione 1	C6 1-Hydroxy-pyridine -2-thione 2	C7 1-Hydroxy-pyridine -2-thione 3	C8 1-Hydroxy-pyridine -2-thione 4	C9 Sodium cyanate 1	C10 Sodium cyanate 2	C11 Sodium cyanate 3	C12 Sodium cyanate 4
D1 Cadmium chloride 1	D2 Cadmium chloride 2	D3 Cadmium chloride 3	D4 Cadmium chloride 4	D5 Iodoacetate 1	D6 Iodoacetate 2	D7 Iodoacetate 3	D8 Iodoacetate 4	D9 Sodium dichromate 1	D10 Sodium dichromate 2	D11 Sodium dichromate 3	D12 Sodium dichromate 4
E1 Cefoxitin 1	E2 Cefoxitin 2	E3 Cefoxitin 3	E4 Cefoxitin 4	E5 Nitrofurantoin 1	E6 Nitrofurantoin 2	E7 Nitrofurantoin 3	E8 Nitrofurantoin 4	E9 Sodium metaborate 1	E10 Sodium metaborate 2	E11 Sodium metaborate 3	E12 Sodium metaborate 4
F1 Chloramphenicol 1	F2 Chloramphenicol 2	F3 Chloramphenicol 3	F4 Chloramphenicol 4	F5 Piperacillin 1	F6 Piperacillin 2	F7 Piperacillin 3	F8 Piperacillin 4	F9 Sodium metavanadate 1	F10 Sodium metavanadate 2	F11 Sodium metavanadate 3	F12 Sodium metavanadate 4
G1 Chelerythrine 1	G2 Chelerythrine 2	G3 Chelerythrine 3	G4 Chelerythrine 4	G5 Carbenicillin 1	G6 Carbenicillin 2	G7 Carbenicillin 3	G8 Carbenicillin 4	G9 Sodium nitrite 1	G10 Sodium nitrite 2	G11 Sodium nitrite 3	G12 Sodium nitrite 4
H1 EGTA 1	H2 EGTA 2	H3 EGTA 3	H4 EGTA 4	H5 Prometrazine 1	H6 Prometrazine 2	H7 Prometrazine 3	H8 Prometrazine 4	H9 Sodium orthovanadate 1	H10 Sodium orthovanadate 2	H11 Sodium orthovanadate 3	H12 Sodium orthovanadate 4

PM15B MicroPlate™

A1 Procaine 1	A2 Procaine 2	A3 Procaine 3	A4 Procaine 4	A5 Guanidine hydrochloride 1	A6 Guanidine hydrochloride 2	A7 Guanidine hydrochloride 3	A8 Guanidine hydrochloride 4	A9 Cefmetazole 1	A10 Cefmetazole 2	A11 Cefmetazole 3	A12 Cefmetazole 4
B1 D-Cycloserine 1	B2 D-Cycloserine 2	B3 D-Cycloserine 3	B4 D-Cycloserine 4	B5 EDTA 1	B6 EDTA 2	B7 EDTA 3	B8 EDTA 4	B9 5,7-Dichloro-8-hydroxy-quinaldine 1	B10 5,7-Dichloro-8-hydroxy-quinaldine 2	B11 5,7-Dichloro-8-hydroxy-quinaldine 3	B12 5,7-Dichloro-8-hydroxy-quinaldine 4
C1 5,7-Dichloro-8-hydroxyquinoline 1	C2 5,7-Dichloro-8-hydroxyquinoline 2	C3 5,7-Dichloro-8-hydroxyquinoline 3	C4 5,7-Dichloro-8-hydroxyquinoline 4	C5 Fusidic acid 1	C6 Fusidic acid 2	C7 Fusidic acid 3	C8 Fusidic acid 4	C9 1,10-Phenanthroline 1	C10 1,10-Phenanthroline 2	C11 1,10-Phenanthroline 3	C12 1,10-Phenanthroline 4
D1 Phleomycin 1	D2 Phleomycin 2	D3 Phleomycin 3	D4 Phleomycin 4	D5 Domiphen bromide 1	D6 Domiphen bromide 2	D7 Domiphen bromide 3	D8 Domiphen bromide 4	D9 Nordihydroguaiaretic acid 1	D10 Nordihydroguaiaretic acid 2	D11 Nordihydroguaiaretic acid 3	D12 Nordihydroguaiaretic acid 4
E1 Alexidine 1	E2 Alexidine 2	E3 Alexidine 3	E4 Alexidine 4	E5 5-Nitro-2-furaldehyde semicarbazone 1	E6 5-Nitro-2-furaldehyde semicarbazone 2	E7 5-Nitro-2-furaldehyde semicarbazone 3	E8 5-Nitro-2-furaldehyde semicarbazone 4	E9 Methyl viologen 1	E10 Methyl viologen 2	E11 Methyl viologen 3	E12 Methyl viologen 4
F1 3, 4-Dimethoxy-benzyl alcohol 1	F2 3, 4-Dimethoxy-benzyl alcohol 2	F3 3, 4-Dimethoxy-benzyl alcohol 3	F4 3, 4-Dimethoxy-benzyl alcohol 4	F5 Oleandomycin 1	F6 Oleandomycin 2	F7 Oleandomycin 3	F8 Oleandomycin 4	F9 Puromycin 1	F10 Puromycin 2	F11 Puromycin 3	F12 Puromycin 4
G1 CCCP 1	G2 CCCP 2	G3 CCCP 3	G4 CCCP 4	G5 Sodium azide 1	G6 Sodium azide 2	G7 Sodium azide 3	G8 Sodium azide 4	G9 Menadione 1	G10 Menadione 2	G11 Menadione 3	G12 Menadione 4
H1 2-Nitroimidazole 1	H2 2-Nitroimidazole 2	H3 2-Nitroimidazole 3	H4 2-Nitroimidazole 4	H5 Hydroxyurea 1	H6 Hydroxyurea 2	H7 Hydroxyurea 3	H8 Hydroxyurea 4	H9 Zinc chloride 1	H10 Zinc chloride 2	H11 Zinc chloride 3	H12 Zinc chloride 4

PM16A MicroPlate™

A1 Cefotaxime 1	A2 Cefotaxime 2	A3 Cefotaxime 3	A4 Cefotaxime 4	A5 Phosphomycin 1	A6 Phosphomycin 2	A7 Phosphomycin 3	A8 Phosphomycin 4	A9 5-Chloro-7-iodo-8-hydroxy-quinoline 1	A10 5-Chloro-7-iodo-8-hydroxy-quinoline 2	A11 5-Chloro-7-iodo-8-hydroxy-quinoline 3	A12 5-Chloro-7-iodo-8-hydroxy-quinoline 4
B1 Norfloxacin 1	B2 Norfloxacin 2	B3 Norfloxacin 3	B4 Norfloxacin 4	B5 Sulfanilamide 1	B6 Sulfanilamide 2	B7 Sulfanilamide 3	B8 Sulfanilamide 4	B9 Trimethoprim 1	B10 Trimethoprim 2	B11 Trimethoprim 3	B12 Trimethoprim 4
C1 Dichlofluanid 1	C2 Dichlofluanid 2	C3 Dichlofluanid 3	C4 Dichlofluanid 4	C5 Protamine sulfate 1	C6 Protamine sulfate 2	C7 Protamine sulfate 3	C8 Protamine sulfate 4	C9 Cetylpyridinium chloride 1	C10 Cetylpyridinium chloride 2	C11 Cetylpyridinium chloride 3	C12 Cetylpyridinium chloride 4
D1 1-Chloro-2,4-dinitrobenzene 1	D2 1-Chloro-2,4-dinitrobenzene 2	D3 1-Chloro-2,4-dinitrobenzene 3	D4 1-Chloro-2,4-dinitrobenzene 4	D5 Diamide 1	D6 Diamide 2	D7 Diamide 3	D8 Diamide 4	D9 Cinoxacin 1	D10 Cinoxacin 2	D11 Cinoxacin 3	D12 Cinoxacin 4
E1 Streptomycin 1	E2 Streptomycin 2	E3 Streptomycin 3	E4 Streptomycin 4	E5 5-Azacytidine 1	E6 5-Azacytidine 2	E7 5-Azacytidine 3	E8 5-Azacytidine 4	E9 Rifamycin SV 1	E10 Rifamycin SV 2	E11 Rifamycin SV 3	E12 Rifamycin SV 4
F1 Potassium tellurite 1	F2 Potassium tellurite 2	F3 Potassium tellurite 3	F4 Potassium tellurite 4	F5 Sodium selenite 1	F6 Sodium selenite 2	F7 Sodium selenite 3	F8 Sodium selenite 4	F9 Aluminum sulfate 1	F10 Aluminum sulfate 2	F11 Aluminum sulfate 3	F12 Aluminum sulfate 4
G1 Chromium chloride 1	G2 Chromium chloride 2	G3 Chromium chloride 3	G4 Chromium chloride 4	G5 Ferric chloride 1	G6 Ferric chloride 2	G7 Ferric chloride 3	G8 Ferric chloride 4	G9 L-Glutamic-g-hydroxamate 1	G10 L-Glutamic-g-hydroxamate 2	G11 L-Glutamic-g-hydroxamate 3	G12 L-Glutamic-g-hydroxamate 4
H1 Glycine hydroxamate 1	H2 Glycine hydroxamate 2	H3 Glycine hydroxamate 3	H4 Glycine hydroxamate 4	H5 Chloramphenicol 1	H6 Chloramphenicol 2	H7 Chloramphenicol 3	H8 Chloramphenicol 4	H9 Sorbic acid 1	H10 Sorbic acid 2	H11 Sorbic acid 3	H12 Sorbic acid 4

BIOLOG Phenotype MicroArrays™

PM17A MicroPlate™

A1 D-Serine 1	A2 D-Serine 2	A3 D-Serine 3	A4 D-Serine 4	A5 β-Chloro-L-alanine hydrochloride 1	A6 β-Chloro-L-alanine hydrochloride 2	A7 β-Chloro-L-alanine hydrochloride 3	A8 β-Chloro-L-alanine hydrochloride 4	A9 Thiosalicylic acid 1	A10 Thiosalicylic acid 2	A11 Thiosalicylic acid 3	A12 Thiosalicylic acid 4
B1 Sodium salicylate 1	B2 Sodium salicylate 2	B3 Sodium salicylate 3	B4 Sodium salicylate 4	B5 Hygromycin B 1	B6 Hygromycin B 2	B7 Hygromycin B 3	B8 Hygromycin B 4	B9 Ethionamide 1	B10 Ethionamide 2	B11 Ethionamide 3	B12 Ethionamide 4
C1 4-Aminopyridine 1	C2 4-Aminopyridine 2	C3 4-Aminopyridine 3	C4 4-Aminopyridine 4	C5 Sulfachloro-pyridazine 1	C6 Sulfachloro-pyridazine 2	C7 Sulfachloro-pyridazine 3	C8 Sulfachloro-pyridazine 4	C9 Sulfamono-methoxine 1	C10 Sulfamono-methoxine 2	C11 Sulfamono-methoxine 3	C12 Sulfamono-methoxine 4
D1 Oxycarboxin 1	D2 Oxycarboxin 2	D3 Oxycarboxin 3	D4 Oxycarboxin 4	D5 3-Amino-1,2,4-triazole 1	D6 3-Amino-1,2,4-triazole 2	D7 3-Amino-1,2,4-triazole 3	D8 3-Amino-1,2,4-triazole 4	D9 Chlorpromazine 1	D10 Chlorpromazine 2	D11 Chlorpromazine 3	D12 Chlorpromazine 4
E1 Niaproof 1	E2 Niaproof 2	E3 Niaproof 3	E4 Niaproof 4	E5 Compound 48/80 1	E6 Compound 48/80 2	E7 Compound 48/80 3	E8 Compound 48/80 4	E9 Sodium tungstate 1	E10 Sodium tungstate 2	E11 Sodium tungstate 3	E12 Sodium tungstate 4
F1 Lithium chloride 1	F2 Lithium chloride 2	F3 Lithium chloride 3	F4 Lithium chloride 4	F5 DL-Methionine hydroxamate 1	F6 DL-Methionine hydroxamate 2	F7 DL-Methionine hydroxamate 3	F8 DL-Methionine hydroxamate 4	F9 Tannic acid 1	F10 Tannic acid 2	F11 Tannic acid 3	F12 Tannic acid 4
G1 Chloramphenicol 1	G2 Chloramphenicol 2	G3 Chloramphenicol 3	G4 Chloramphenicol 4	G5 Cefamandole sodium 1	G6 Cefamandole sodium 2	G7 Cefamandole sodium 3	G8 Cefamandole sodium 4	G9 Cefoperazone 1	G10 Cefoperazone 2	G11 Cefoperazone 3	G12 Cefoperazone 4
H1 Cefsulodin 1	H2 Cefsulodin 2	H3 Cefsulodin 3	H4 Cefsulodin 4	H5 Caffeine 1	H6 Caffeine 2	H7 Caffeine 3	H8 Caffeine 4	H9 Phenylarsine oxide 1	H10 Phenylarsine oxide 2	H11 Phenylarsine oxide 3	H12 Phenylarsine oxide 4

PM18C MicroPlate™

A1 Ketoprofen 1	A2 Ketoprofen 2	A3 Ketoprofen 3	A4 Ketoprofen 4	A5 Sodium pyrophosphate decahydrate 1	A6 Sodium pyrophosphate decahydrate 2	A7 Sodium pyrophosphate decahydrate 3	A8 Sodium pyrophosphate decahydrate 4	A9 Thiampheni col 1	A10 Thiamphenicol 2	A11 Thiamphenicol 3	A12 Thiamphenicol 4
B1 Trifluoperazine 1	B2 Trifluoperazine 2	B3 Trifluoperazine 3	B4 Trifluoperazine 4	B5 Pipemidic Acid 1	B6 Pipemidic Acid 2	B7 Pipemidic Acid 3	B8 Pipemidic Acid 4	B9 Azathioprine 1	B10 Azathioprine 2	B11 Azathioprine 3	B12 Azathioprine 4
C1 Poly-L-lysine 1	C2 Poly-L-lysine 2	C3 Poly-L-lysine 3	C4 Poly-L-lysine 4	C5 Sulfisoxazole 1	C6 Sulfisoxazole 2	C7 Sulfisoxazole 3	C8 Sulfisoxazole 4	C9 Pentachloro-phenol 1	C10 Pentachloro-phenol 2	C11 Pentachloro-phenol 3	C12 Pentachloro-phenol 4
D1 Sodium m-arsenite 1	D2 Sodium m-arsenite 2	D3 Sodium m-arsenite 3	D4 Sodium m-arsenite 4	D5 Sodium bromate 1	D6 Sodium bromate 2	D7 Sodium bromate 3	D8 Sodium bromate 4	D9 Lidocaine 1	D10 Lidocaine 2	D11 Lidocaine 3	D12 Lidocaine 4
E1 Sodium metasilicate 1	E2 Sodium metasilicate 2	E3 Sodium metasilicate 3	E4 Sodium metasilicate 4	E5 Sodium m-periodate 1	E6 Sodium m-periodate 2	E7 Sodium m-periodate 3	E8 Sodium m-periodate 4	F9 Antimony (III) chloride 1	E10 Antimony (III) chloride 2	E11 Antimony (III) chloride 3	E12 Antimony (III) chloride 4
F1 Semicarbazide 1	F2 Semicarbazide 2	F3 Semicarbazide 3	F4 Semicarbazide 4	F5 Thiabendazole 1	F6 Thiabendazole 2	F7 Thiabendazole 3	F8 Thiabendazole 4	F9 Aztreonam 1	F10 Aztreonam 2	F11 Aztreonam 3	F12 Aztreonam 4
G1 Tricine 1	G2 Tricine 2	G3 Tricine 3	G4 Tricine 4	G5 2,5-Diamino-1,2,4-triazole (Guanazole) 1	G6 2,5-Diamino-1,2,4-triazole (Guanazole) 2	G7 2,5-Diamino-1,2,4-triazole (Guanazole) 3	G8 2,5-Diamino-1,2,4-triazole (Guanazole) 4	G9 Myristin 1	G10 Myristin 2	G11 Myristin 3	G12 Myristin 4
H1 5-Fluoro-2'-deoxyuridine 1	H2 5-Fluoro-2'-deoxyuridine 2	H3 5-Fluoro-2'-deoxyuridine 3	H4 5-Fluoro-2'-deoxyuridine 4	H5 2-Phenylphenol 1	H6 2-Phenylphenol 2	H7 2-Phenylphenol 3	H8 2-Phenylphenol 4	H9 Plumbagin 1	H10 Plumbagin 2	H11 Plumbagin 3	H12 Plumbagin 4

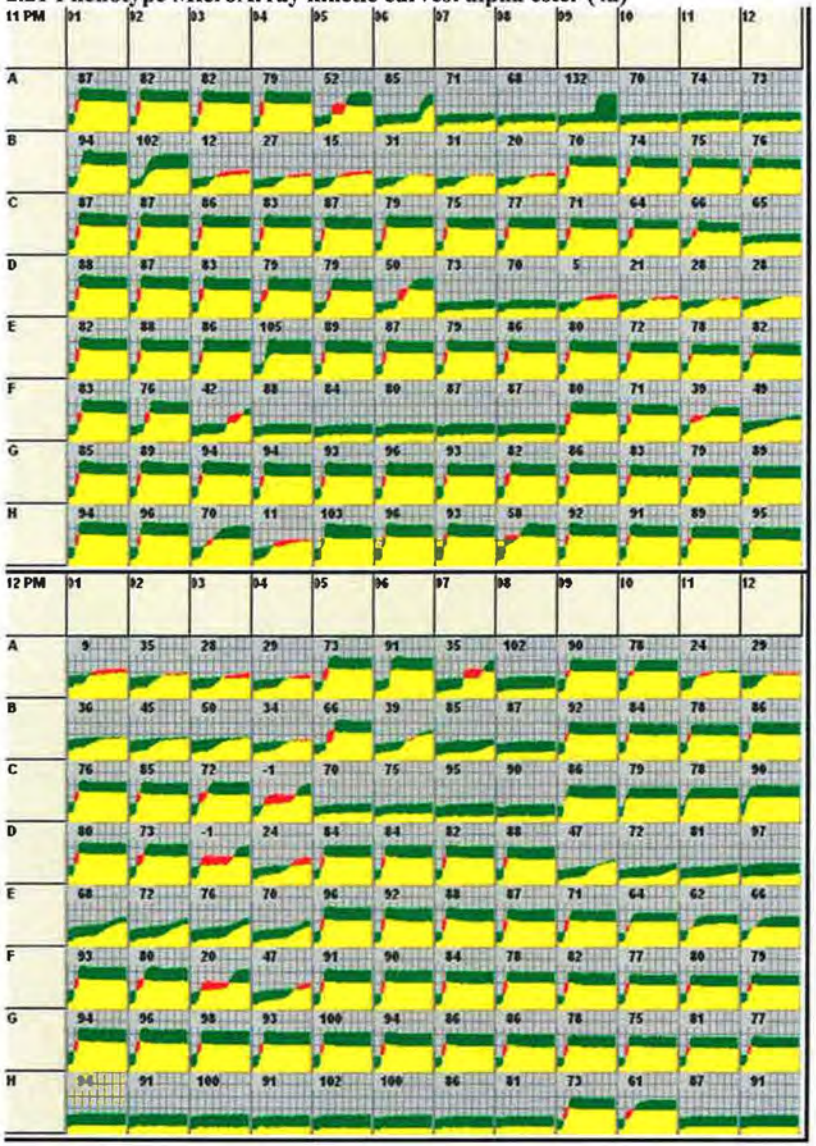
PM19 MicroPlate™

A1 Josamycin 1	A2 Josamycin 2	A3 Josamycin 3	A4 Josamycin 4	A5 Gallic acid 1	A6 Gallic acid 2	A7 Gallic acid 3	A8 Gallic acid 4	A9 Coumarin 1	A10 Coumarin 2	A11 Coumarin 3	A12 Coumarin 4
B1 Methyltrioctyl-ammonium chloride 1	B2 Methyltrioctyl-ammonium chloride 2	B3 Methyltrioctyl-ammonium chloride 3	B4 Methyltrioctyl-ammonium chloride 4	B5 Hemimin 1	B6 Hemimin 2	B7 Hemimin 3	B8 Hemimin 4	B9 2,4-Dinitrophenol 1	B10 2,4-Dinitrophenol 2	B11 2,4-Dinitrophenol 3	B12 2,4-Dinitrophenol 4
C1 Chlorhexidine 1	C2 Chlorhexidine 2	C3 Chlorhexidine 3	C4 Chlorhexidine 4	C5 Umbelliferone 1	C6 Umbelliferone 2	C7 Umbelliferone 3	C8 Umbelliferone 4	C9 Cinnamic acid 1	C10 Cinnamic acid 2	C11 Cinnamic acid 3	C12 Cinnamic acid 4
D1 Disulphiram 1	D2 Disulphiram 2	D3 Disulphiram 3	D4 Disulphiram 4	D5 Iodonitro Tetrazolium Violet 1	D6 Iodonitro Tetrazolium Violet 2	D7 Iodonitro Tetrazolium Violet 3	D8 Iodonitro Tetrazolium Violet 4	D9 Phenyl- methyl-sulfonyl-fluoride (PMSF) 1	D10 Phenyl- methyl-sulfonyl-fluoride (PMSF) 2	D11 Phenyl- methyl-sulfonyl-fluoride (PMSF) 3	D12 Phenyl- methyl-sulfonyl-fluoride (PMSF) 4
E1 FCCP 1	E2 FCCP 2	E3 FCCP 3	E4 FCCP 4	E5 D,L-Thiocitic Acid 1	E6 D,L-Thiocitic Acid 2	E7 D,L-Thiocitic Acid 3	E8 D,L-Thiocitic Acid 4	E9 Lawsone 1	E10 Lawsone 2	E11 Lawsone 3	E12 Lawsone 4
F1 Phenethicillin 1	F2 Phenethicillin 2	F3 Phenethicillin 3	F4 Phenethicillin 4	F5 Bacitracin B 1	F6 Bacitracin B 2	F7 Bacitracin B 3	F8 Bacitracin B 4	F9 Sodium caprylate 1	F10 Sodium caprylate 2	F11 Sodium caprylate 3	F12 Sodium caprylate 4
G1 Lauryl sulfobetaine 1	G2 Lauryl sulfobetaine 2	G3 Lauryl sulfobetaine 3	G4 Lauryl sulfobetaine 4	G5 Dihydro-streptomycin 1	G6 Dihydro-streptomycin 2	G7 Dihydro-streptomycin 3	G8 Dihydro-streptomycin 4	G9 Hydroxylamine 1	G10 Hydroxylamine 2	G11 Hydroxylamine 3	G12 Hydroxylamine 4
H1 Hexammine cobalt (III) chloride 1	H2 Hexammine cobalt (III) chloride 2	H3 Hexammine cobalt (III) chloride 3	H4 Hexammine cobalt (III) chloride 4	H5 Thioglycerol 1	H6 Thioglycerol 2	H7 Thioglycerol 3	H8 Thioglycerol 4	H9 Polymyxin B 1	H10 Polymyxin B 2	H11 Polymyxin B 3	H12 Polymyxin B 4

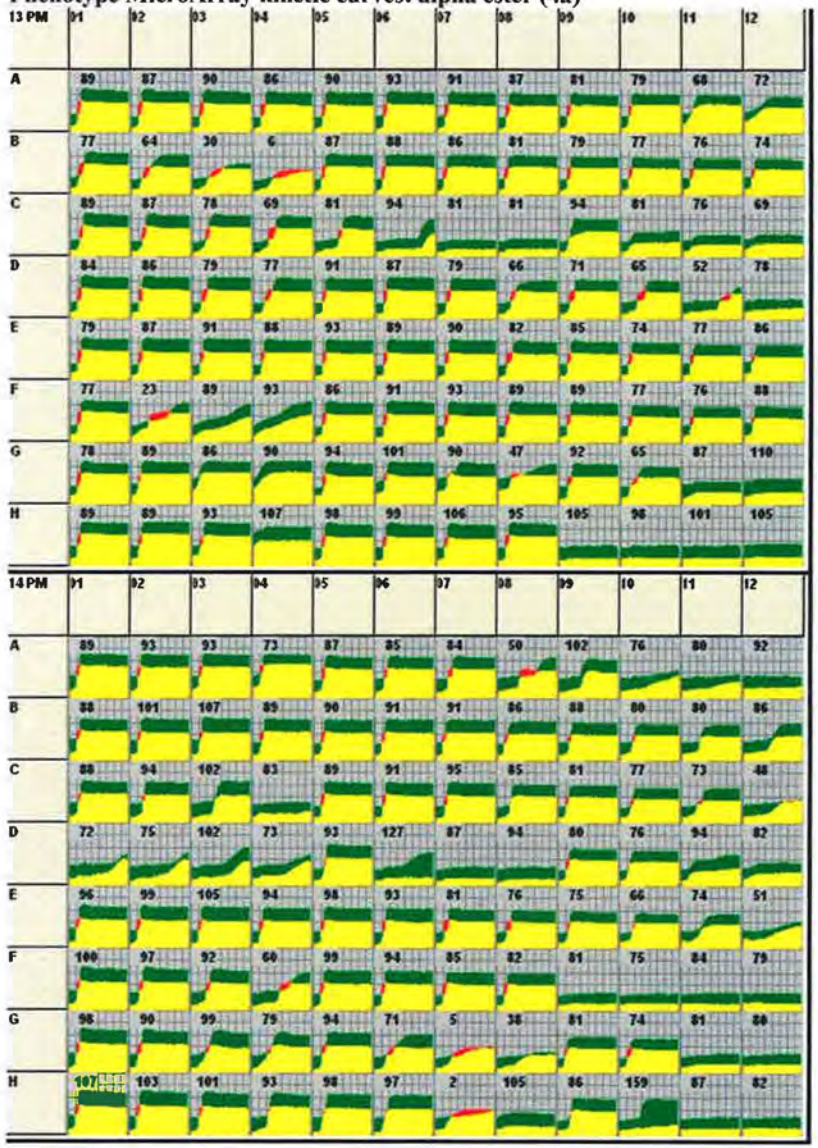
PM20B MicroPlate™

A1 Amitriptyline 1	A2 Amitriptyline 2	A3 Amitriptyline 3	A4 Amitriptyline 4	A5 Apramycin 1	A6 Apramycin 2	A7 Apramycin 3	A8 Apramycin 4	A9 Benzamidine 1	A10 Benzamidine 2	A11 Benzamidine 3	A12 Benzamidine 4
B1 Orphenadrine 1	B2 Orphenadrine 2	B3 Orphenadrine 3	B4 Orphenadrine 4	B5 D,L-Propranolol 1	B6 D,L-Propranolol 2	B7 D,L-Propranolol 3	B8 D,L-Propranolol 4	B9 Tetrazolium violet 1	B10 Tetrazolium violet 2	B11 Tetrazolium violet 3	B12 Tetrazolium violet 4
C1 Thioridazine 1	C2 Thioridazine 2	C3 Thioridazine 3	C4 Thioridazine 4	C5 Atropine 1	C6 Atropine 2	C7 Atropine 3	C8 Atropine 4	C9 Oxiconazole 1	C10 Oxiconazole 2	C11 Oxiconazole 3	C12 Oxiconazole 4
D1 Proflavine 1	D2 Proflavine 2	D3 Proflavine 3	D4 Proflavine 4	D5 Ciprofloxacin 1	D6 Ciprofloxacin 2	D7 Ciprofloxacin 3	D8 Ciprofloxacin 4	D9 18-Crown-6 ether 1	D10 18-Crown-6 ether 2	D11 18-Crown-6 ether 3	D12 18-Crown-6 ether 4
E1 Crystal violet 1	E2 Crystal violet 2	E3 Crystal violet 3	E4 Crystal violet 4	E5 Dodine 1	E6 Dodine 2	E7 Dodine 3	E8 Dodine 4	E9 Hexachlorophene 1	E10 Hexachlorophene 2	E11 Hexachlorophene 3	E12 Hexachlorophene 4
F1 4-Hydroxy-coumarin 1	F2 4-Hydroxy-coumarin 2	F3 4-Hydroxy-coumarin 3	F4 4-Hydroxy-coumarin 4	F5 Oxytetracycline 1	F6 Oxytetracycline 2	F7 Oxytetracycline 3	F8 Oxytetracycline 4	F9 Pristinol 1	F10 Pristinol 2	F11 Pristinol 3	F12 Pristinol 4
G1 Captan 1	G2 Captan 2	G3 Captan 3	G4 Captan 4	G5 3,5-Dinitro-benzene 1	G6 2,5-Dinitro-benzene 2	G7 2,5-Dinitro-benzene 3	G8 3,5-Dinitro-benzene 4	G9 8-Hydroxy-quinoline 1	G10 8-Hydroxy-quinoline 2	G11 8-Hydroxy-quinoline 3	G12 8-Hydroxy-quinoline 4
H1 Patulin 1	H2 Patulin 2	H3 Patulin 3	H4 Patulin 4	H5 Tolylfluanid 1	H6 Tolylfluanid 2	H7 Tolylfluanid 3	H8 Tolylfluanid 4	H9 Troleandomycin 1	H10 Troleandomycin 2	H11 Troleandomycin 3	H12 Troleandomycin 4

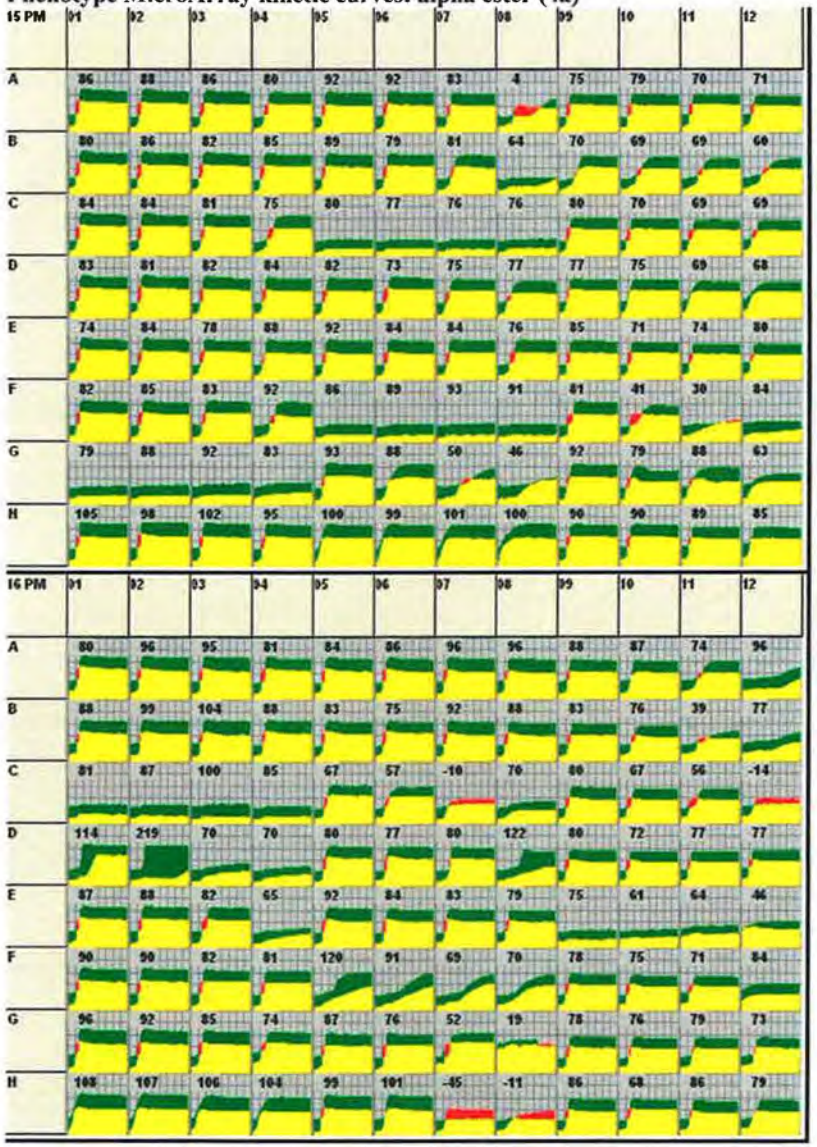
2.21 Phenotype MicroArray kinetic curves: alpha ester (4a)



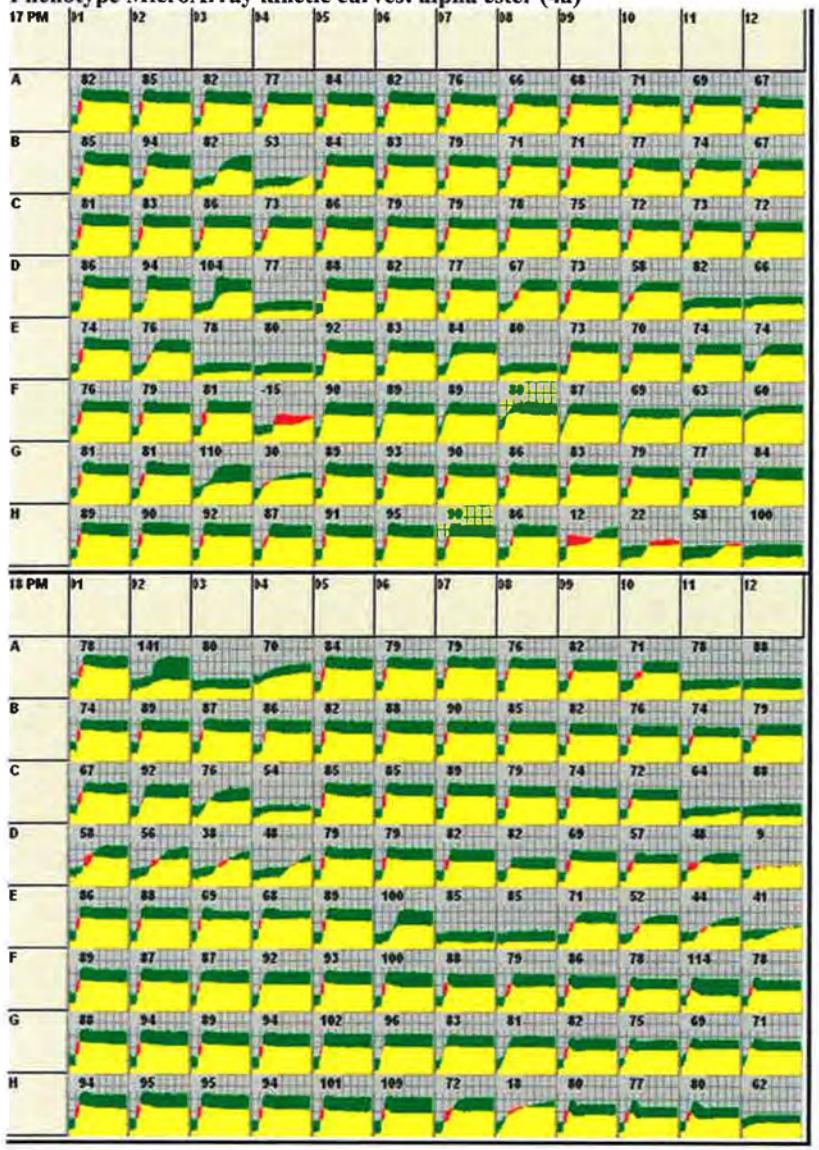
Phenotype MicroArray kinetic curves: alpha ester (4a)



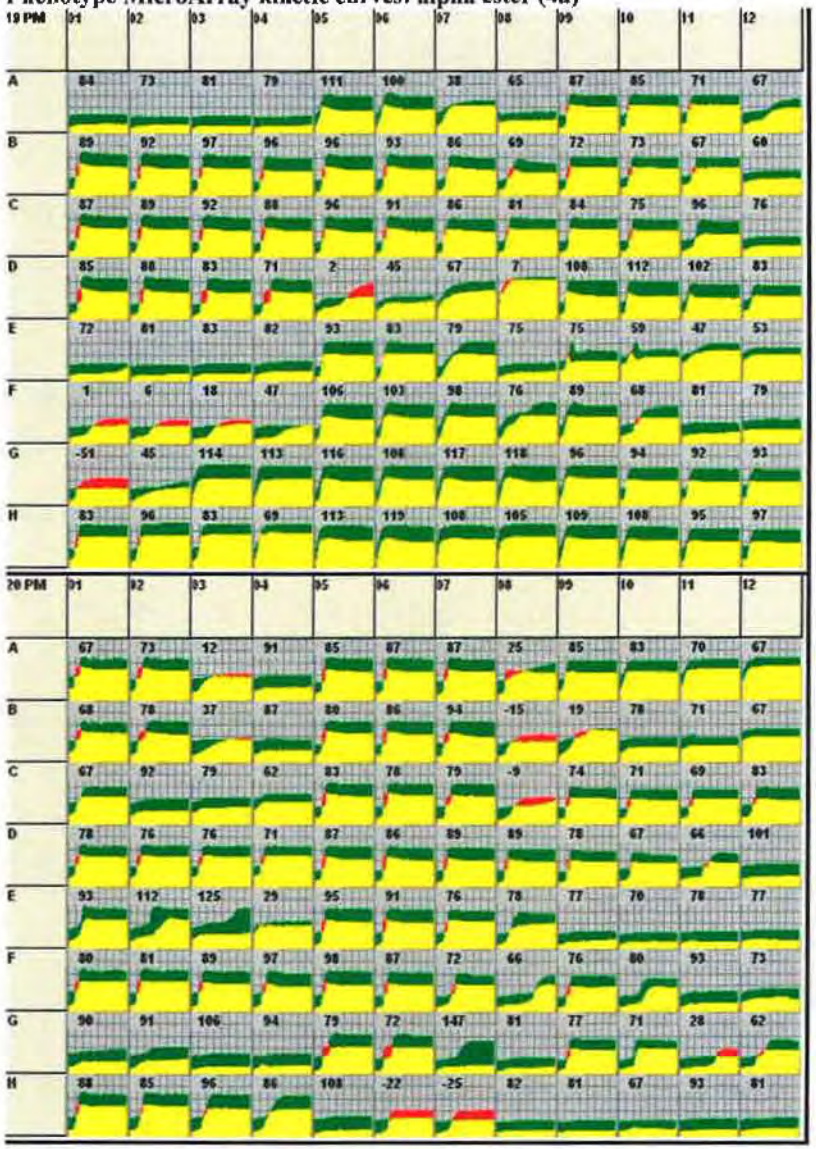
Phenotype MicroArray kinetic curves: alpha ester (4a)



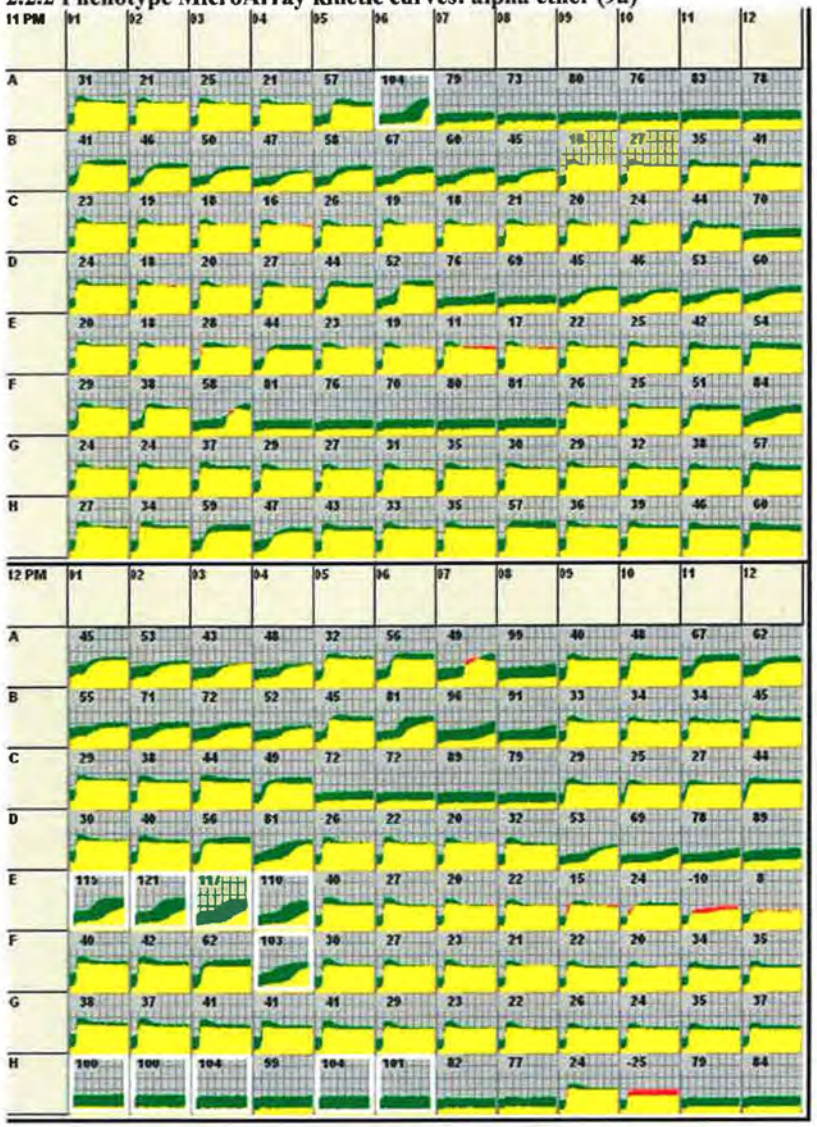
Phenotype MicroArray kinetic curves: alpha ester (4a)

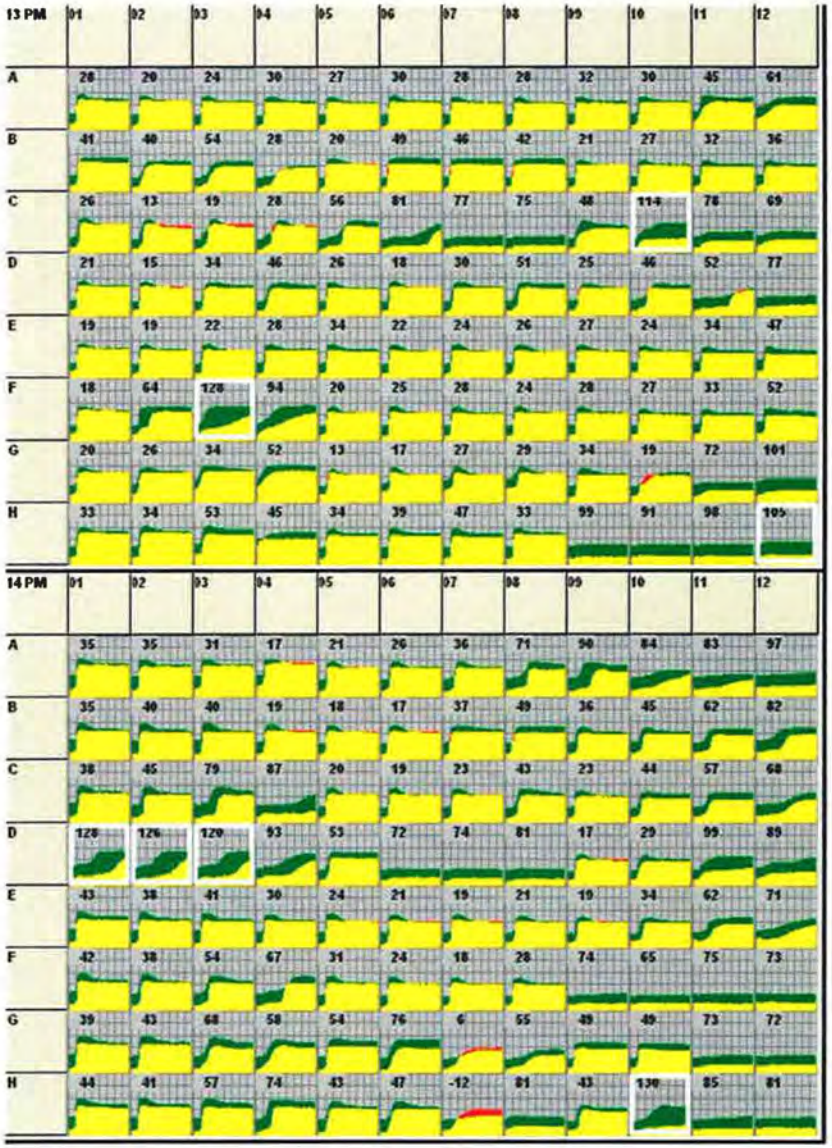


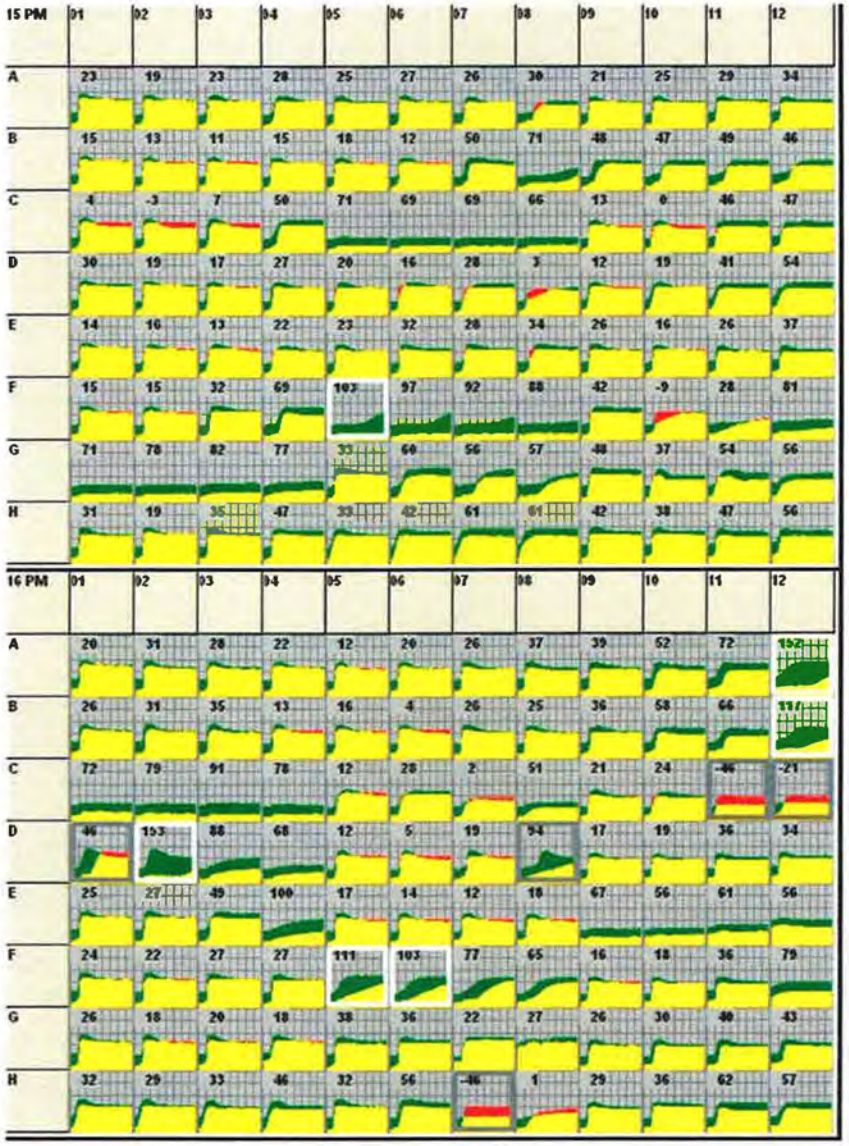
Phenotype MicroArray kinetic curves: alpha ester (4a)

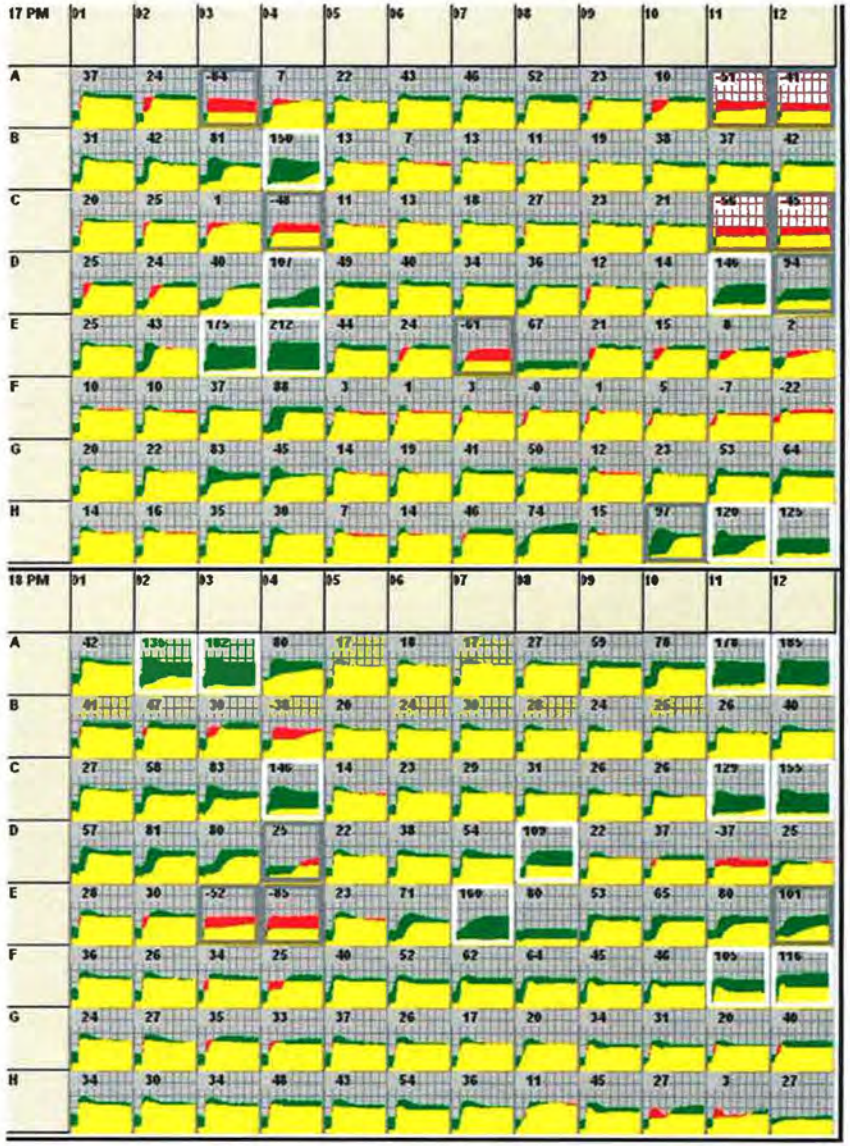


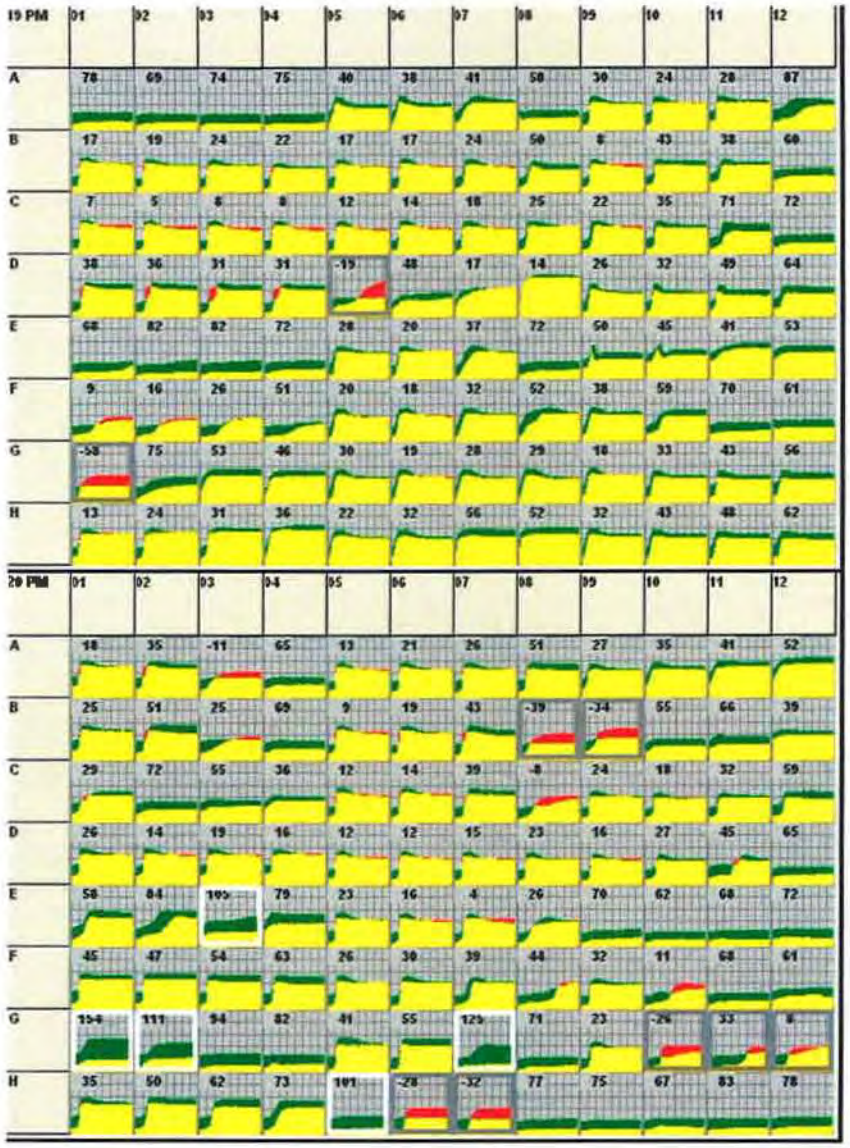
2.2.2 Phenotype MicroArray kinetic curves: alpha ether (9a)



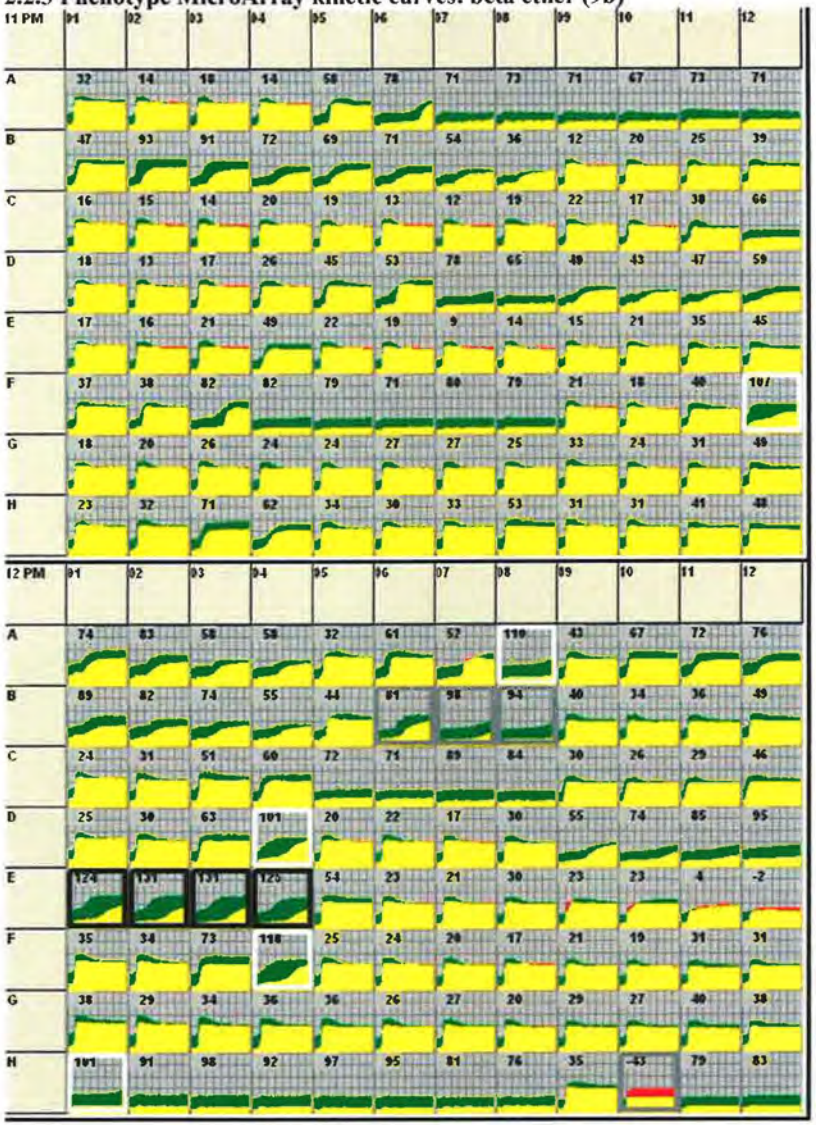


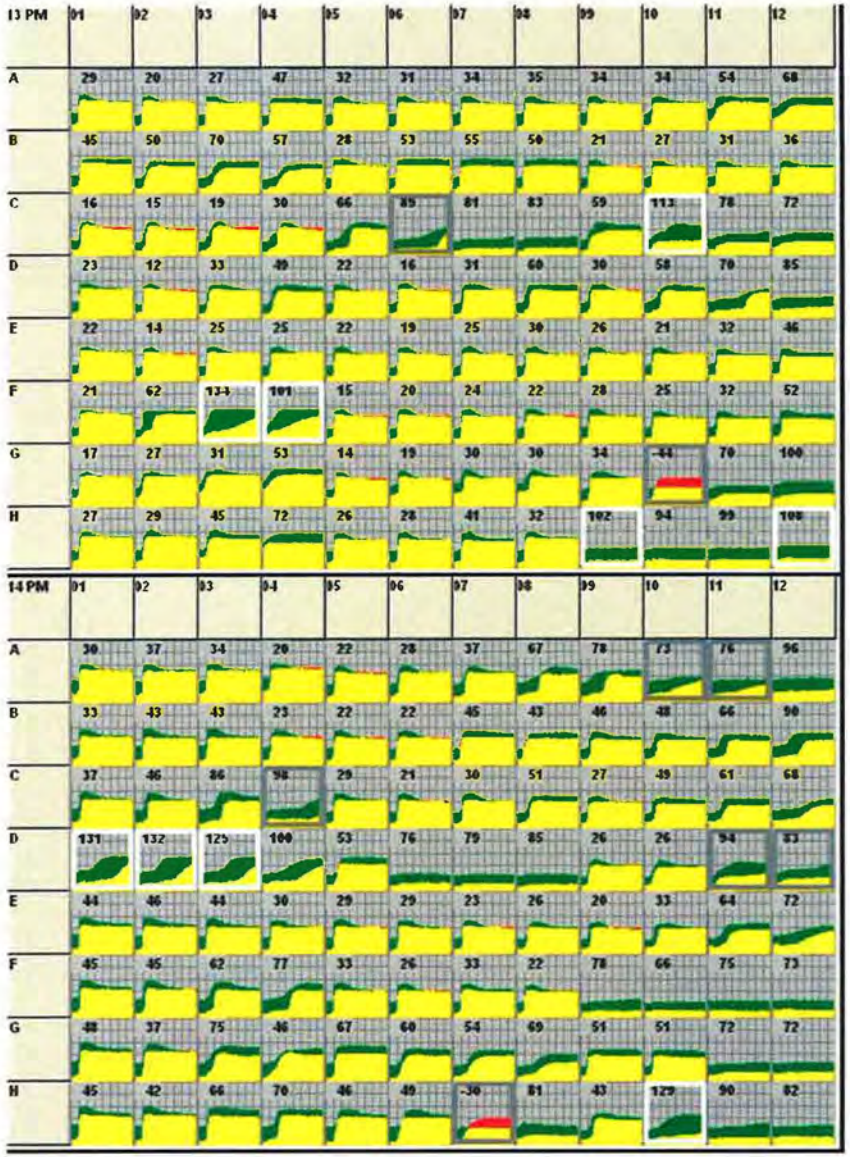

15 PM
16 PM

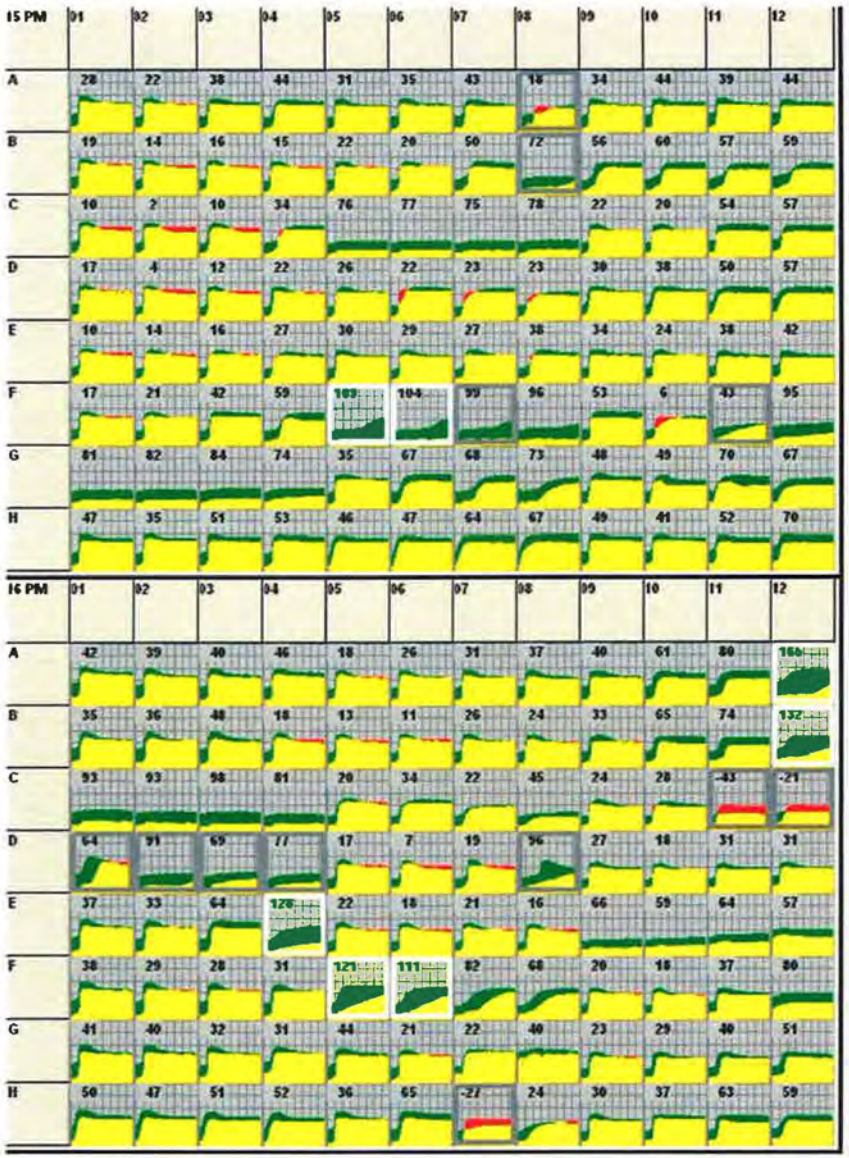

17 PM
18 PM

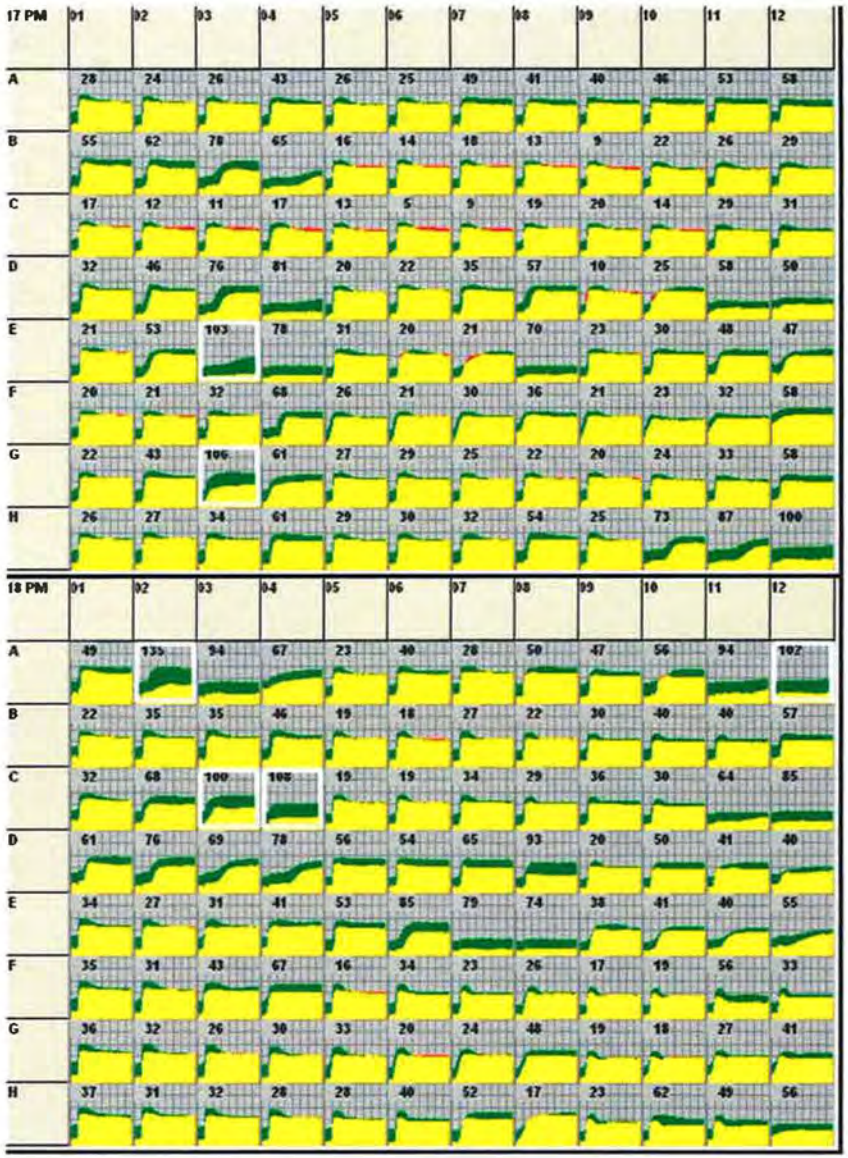

19 PM
01 02 03 04 05 06 07 08 09 10 11 12
A 78 69 74 75 40 38 41 58 30 24 28 87
B 17 19 24 22 17 17 24 50 8 43 38 60
C 7 5 6 8 12 14 18 25 22 35 71 72
D 38 36 31 31 -19 48 17 14 26 32 49 64
E 68 82 82 72 28 20 37 72 50 45 41 53
F 9 16 26 51 20 18 32 52 38 59 70 61
G -58 75 53 46 30 19 28 29 18 33 43 56
H 13 24 31 36 22 32 56 52 32 43 48 62
20 PM
01 02 03 04 05 06 07 08 09 10 11 12
A 18 35 -11 65 13 21 26 51 27 35 41 52
B 25 51 25 69 9 19 43 -39 -34 55 66 39
C 29 72 55 36 12 14 39 -8 24 18 32 59
D 26 14 19 16 12 12 15 23 16 27 45 65
E 58 84 105 79 23 16 4 26 70 62 68 72
F 45 47 54 63 26 30 39 48 32 11 68 61
G 104 111 94 82 41 55 125 71 23 -24 33 8
H 35 50 62 73 101 -28 -32 77 75 67 83 78

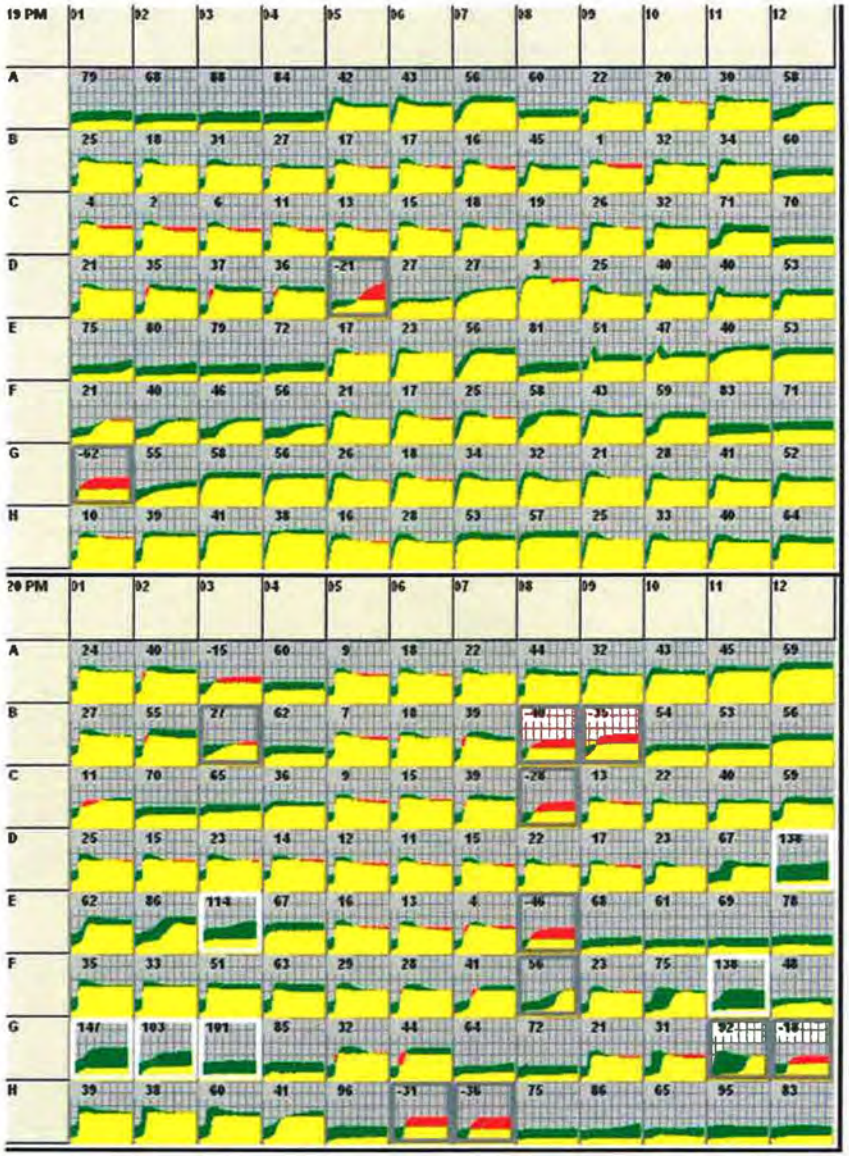
2.2.3 Phenotype MicroArray kinetic curves: beta ether (9b)



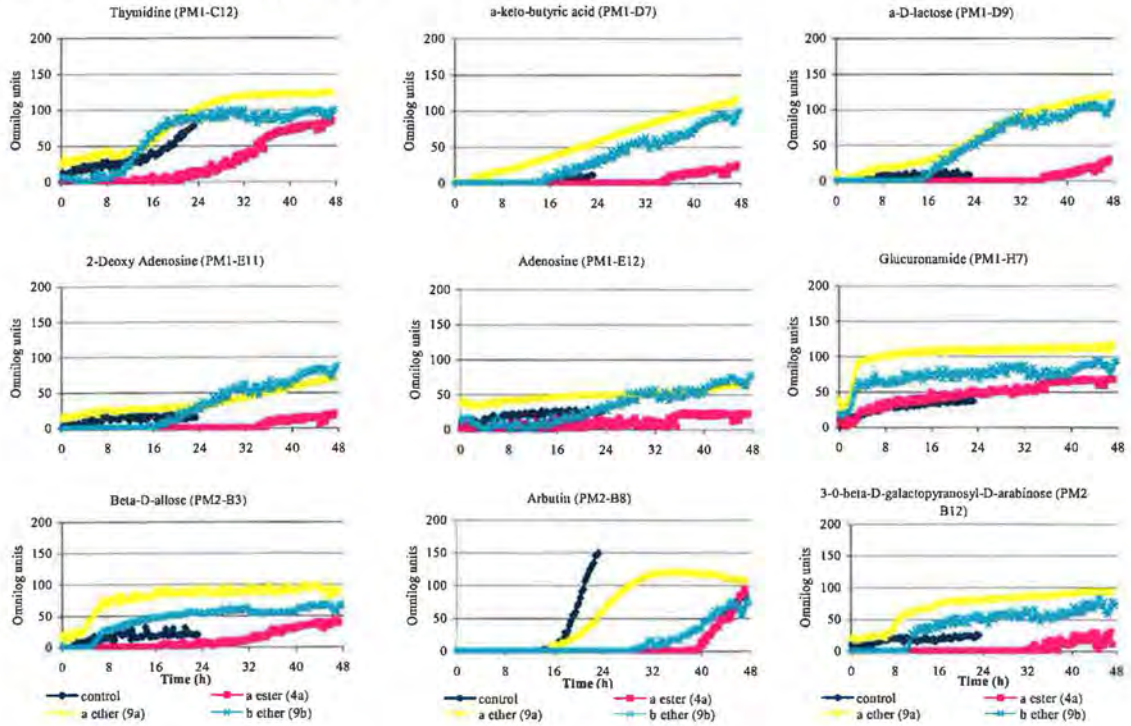


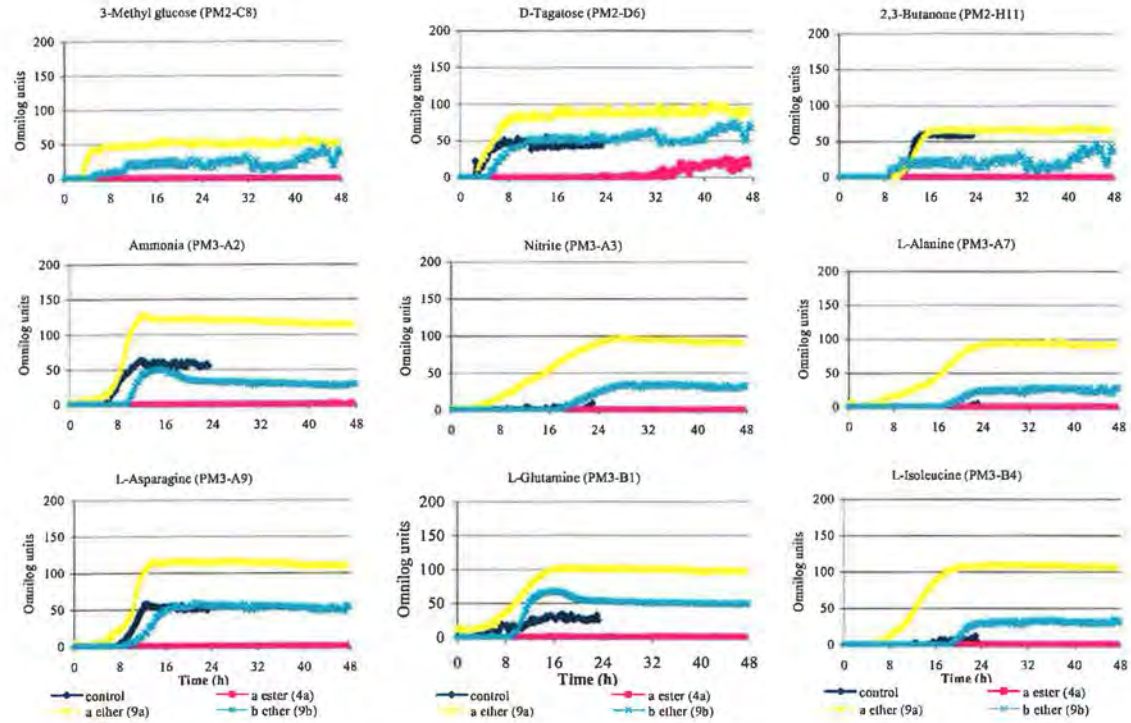

15 PM
16 PM

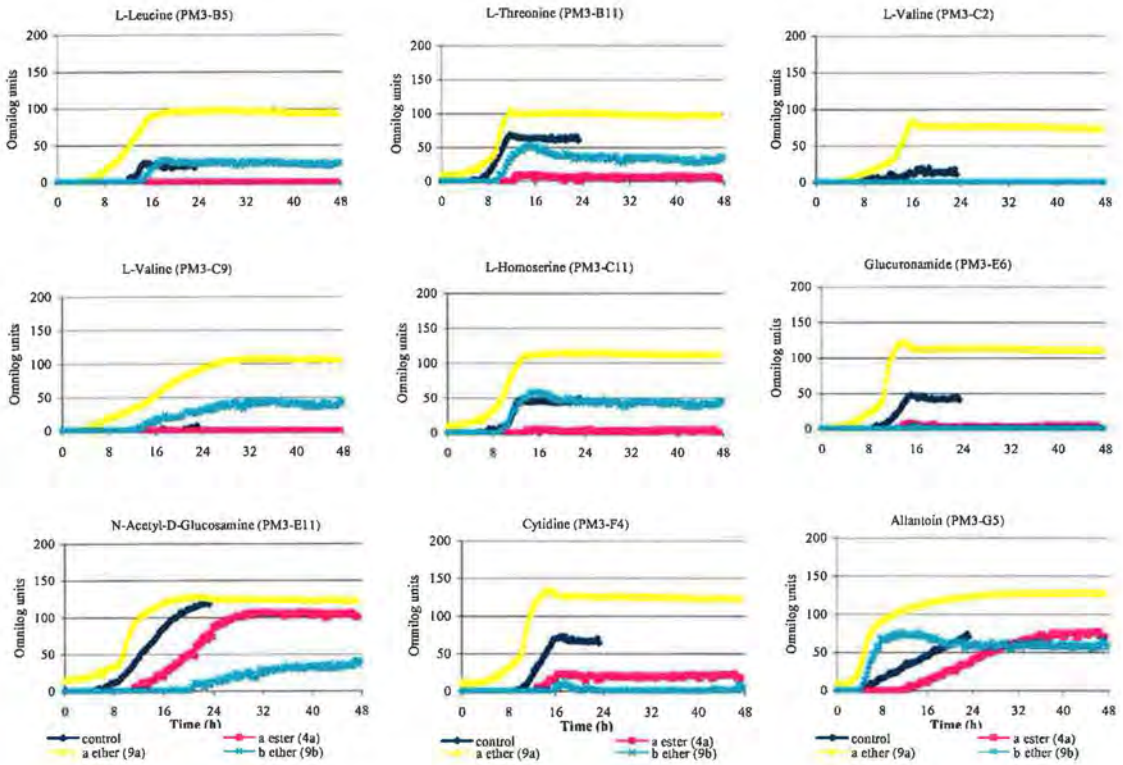

17 PM
18 PM

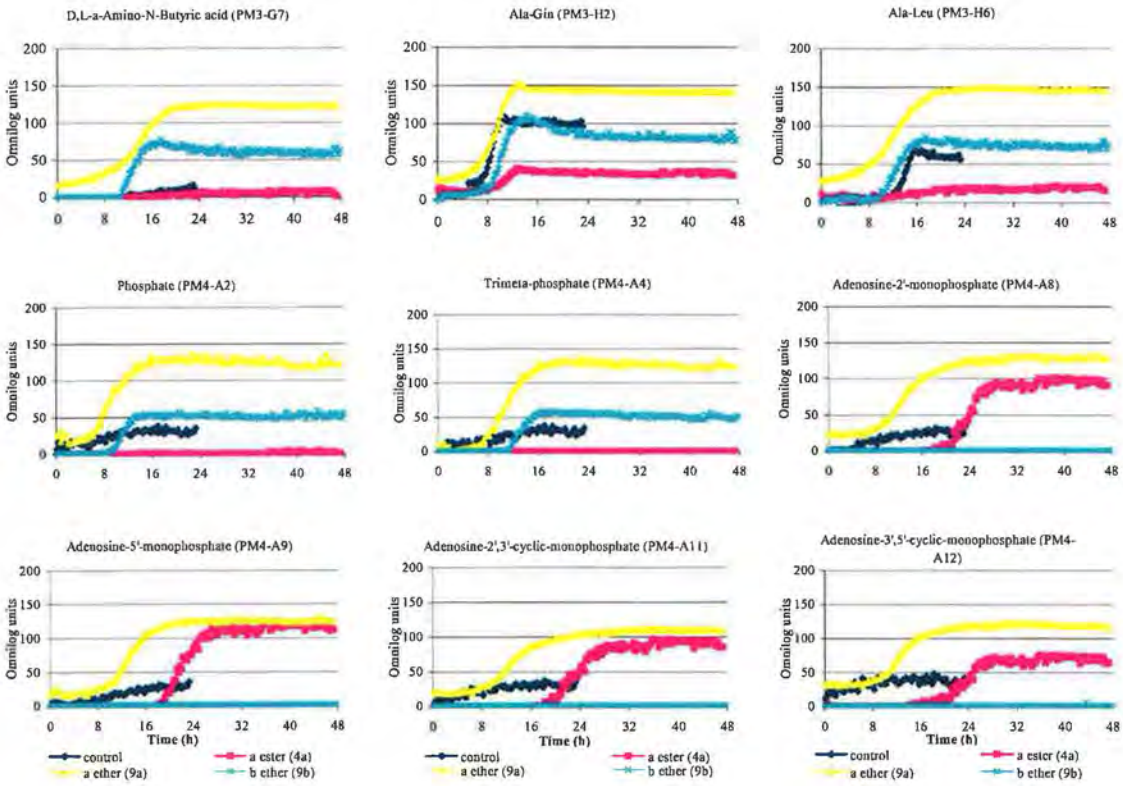

19 PM
01 02 03 04 05 06 07 08 09 10 11 12
A 79 68 88 84 42 43 56 60 22 20 30 58
B 25 18 31 27 17 17 16 45 1 32 34 60
C -4 2 6 11 13 15 18 19 26 32 71 70
D 21 35 37 36 -21 27 27 3 25 40 40 53
E 75 80 79 72 17 23 56 81 51 47 40 53
F 21 40 46 56 21 17 25 58 43 59 83 71
G -62 55 58 56 26 18 34 32 21 28 41 52
H 10 39 41 38 16 28 53 57 25 33 40 64
20 PM
01 02 03 04 05 06 07 08 09 10 11 12
A 24 40 -15 60 9 18 22 44 32 43 45 59
B 27 55 27 62 7 18 39 54 53 56
C 11 70 65 36 9 15 39 13 22 40 59
D 25 15 23 14 12 11 15 22 17 23 67 138
E 62 86 114 67 16 13 4 68 61 69 78
F 35 33 51 63 29 28 41 56 23 75 138 48
G 147 103 101 85 32 44 64 72 21 31 92
H 39 38 60 41 96 -31 75 86 65 95 83

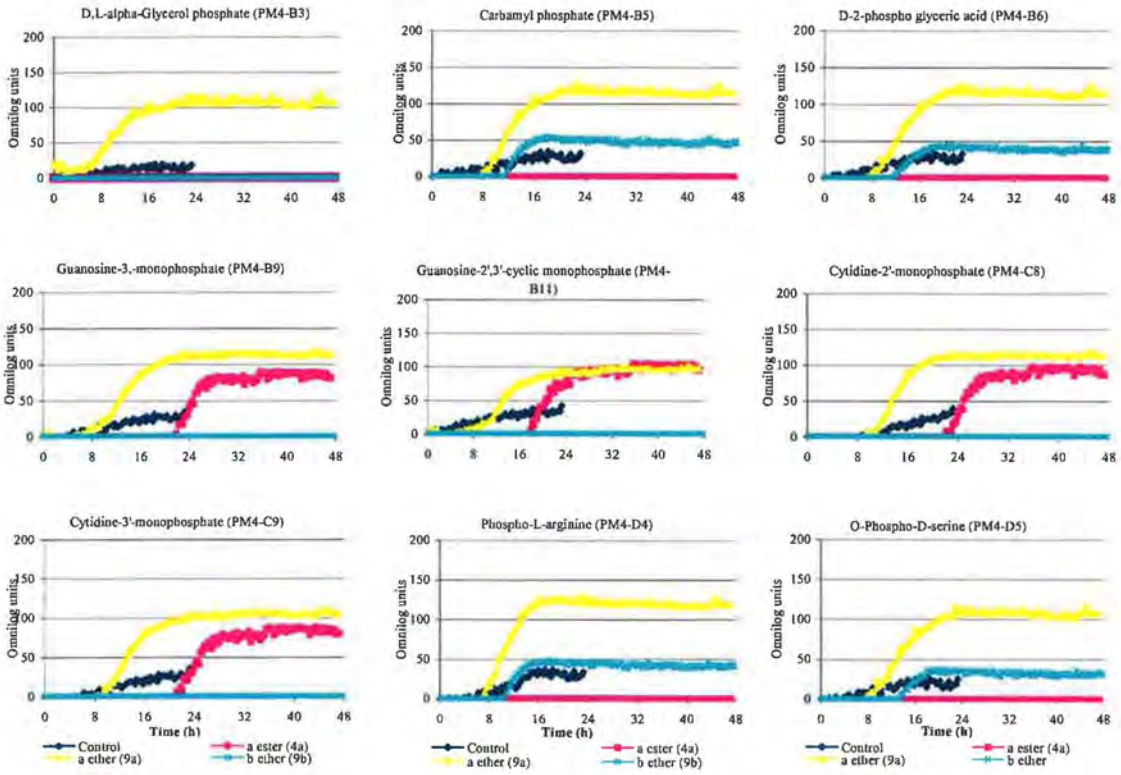
2.3 Phenotype MicroArray hits plates 1-10

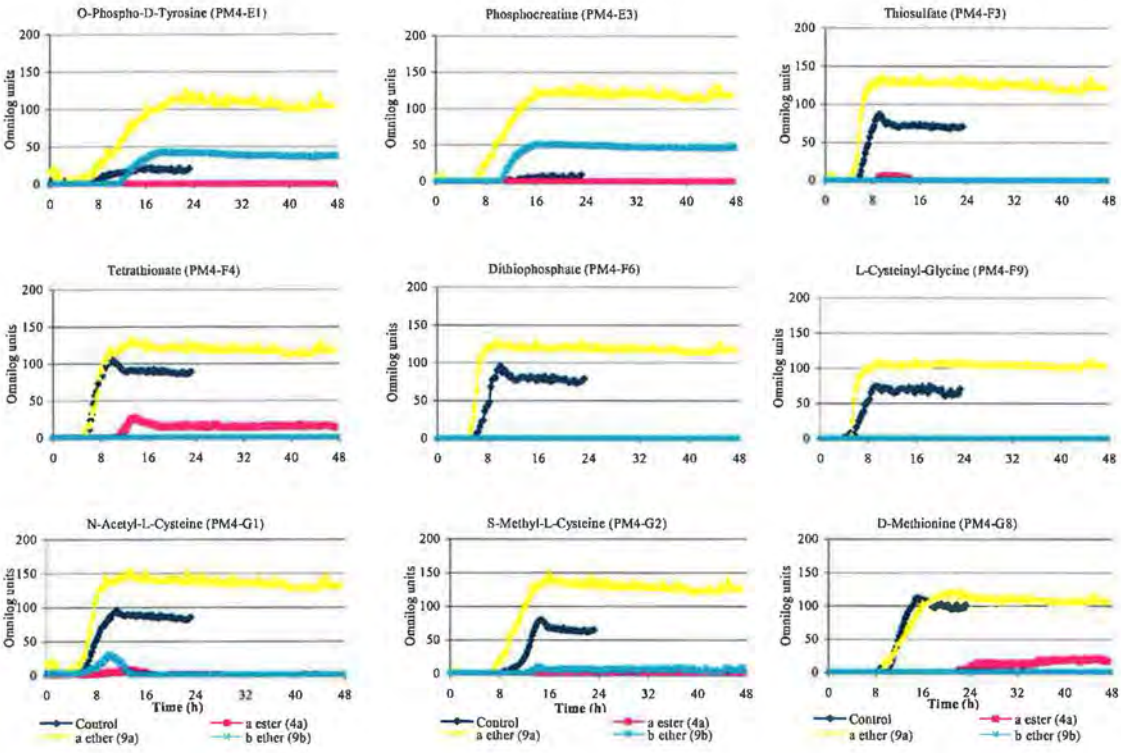


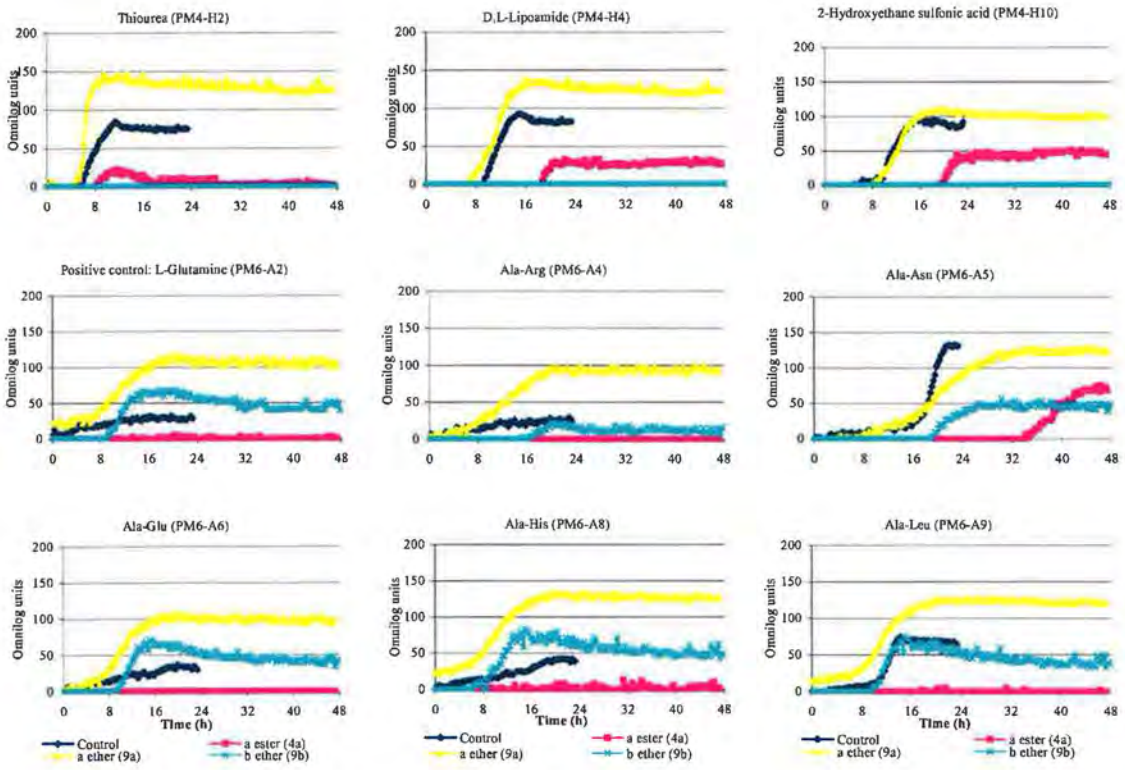


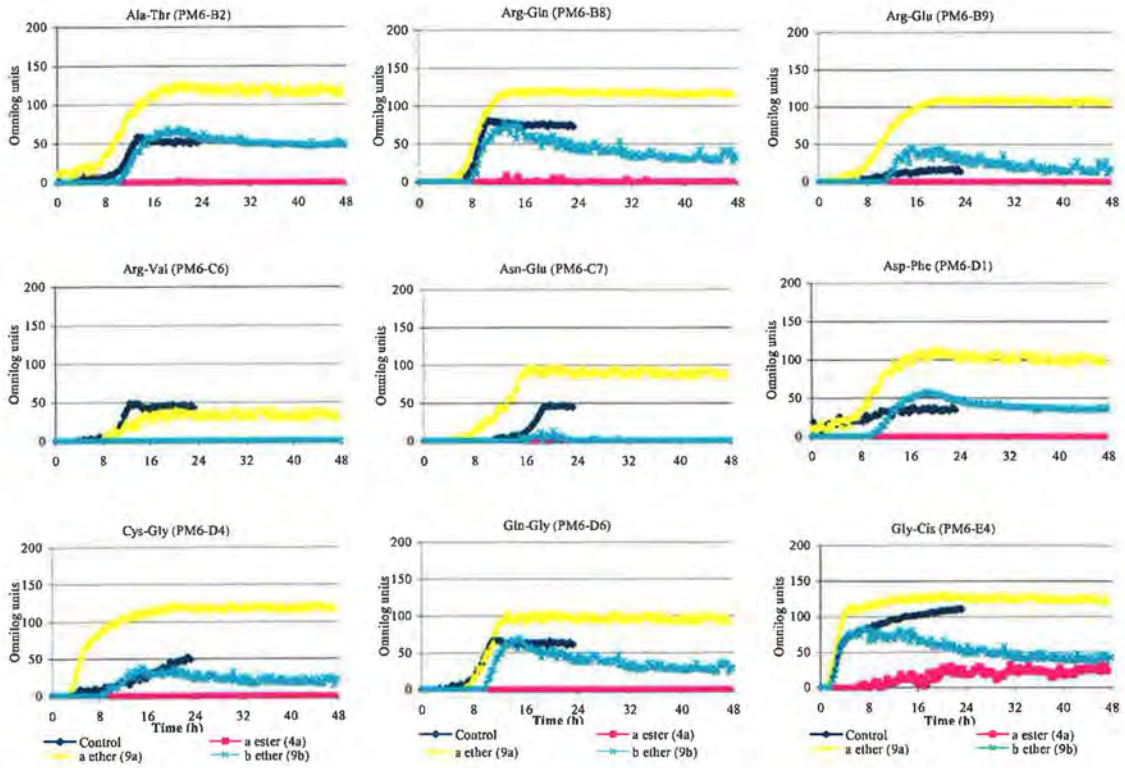

L-Leucine (PM3-B5)
L-Threonine (PM3-B11)
L-Valine (PM3-C2)
L-Valine (PM3-C9)
L-Homoserine (PM3-C11)
Glucuronamide (PM3-E6)
N-Acetyl-D-Glucosamine (PM3-E11)
Cytidine (PM3-F4)
Allantoin (PM3-G5)
Omnilog units
Time (h)
control
a ether (9a)
a ester (4a)
b ether (9b)

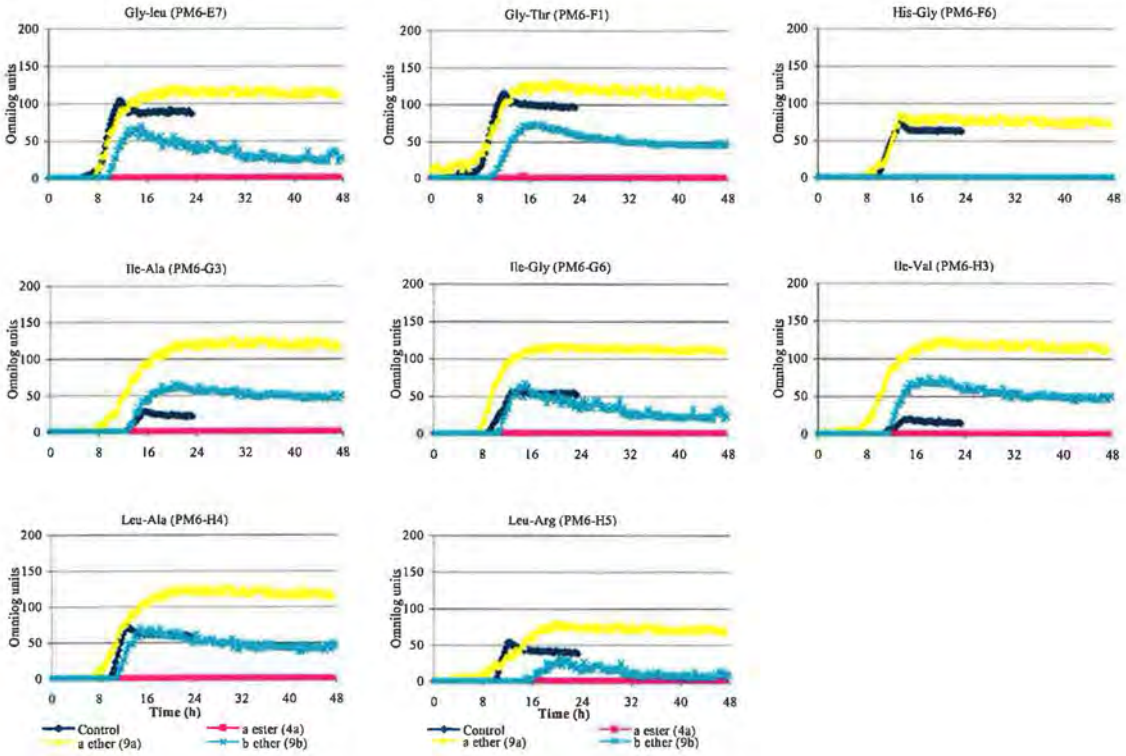

D,L-a-Amino-N-Butyric acid (PM3-G7)
Ala-Gln (PM3-H2)
Ala-Leu (PM3-H6)
Phosphate (PM4-A2)
Trimeta-phosphate (PM4-A4)
Adenosine-2'-monophosphate (PM4-A8)
Adenosine-5'-monophosphate (PM4-A9)
Adenosine-2',3'-cyclic-monophosphate (PM4-A11)
Adenosine-3',5'-cyclic-monophosphate (PM4-A12)
Omnilog units
Time (h)
control
a ether (9a)
a ester (4a)
b ether (9b)

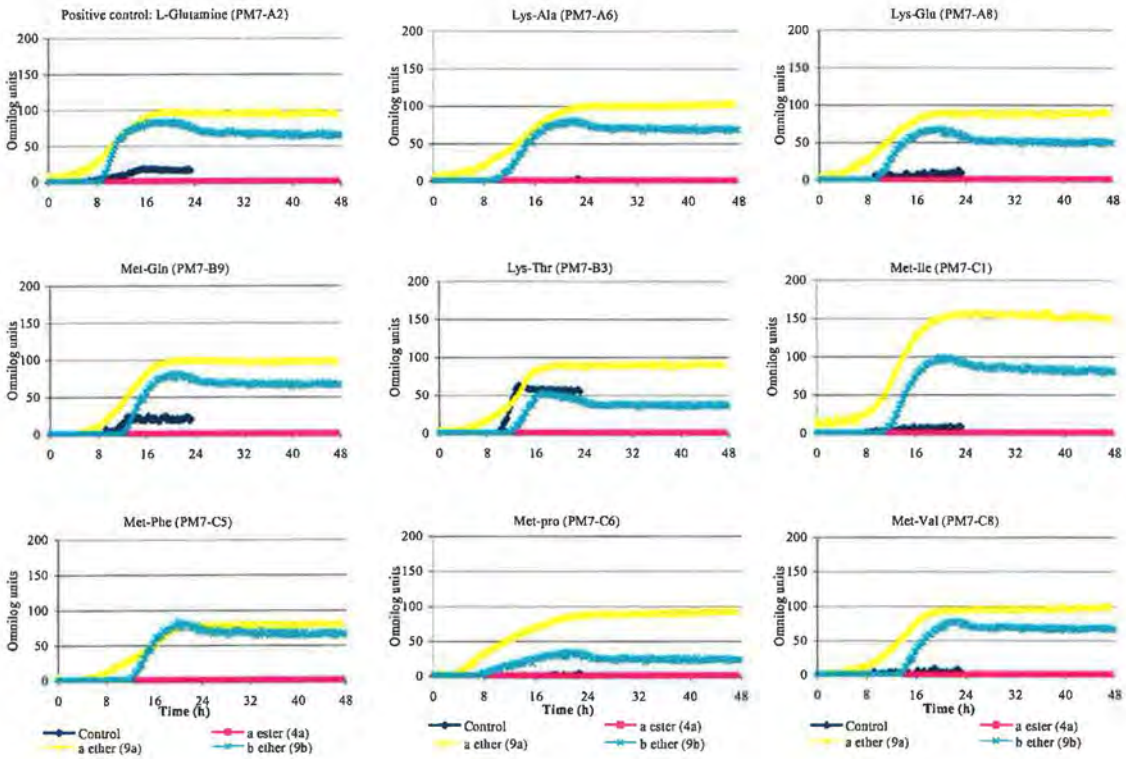

D,L-alpha-Glycerol phosphate (PM4-B3)
Carbamyl phosphate (PM4-B5)
D-2-phospho glyceric acid (PM4-B6)
Guanosine-3,-monophosphate (PM4-B9)
Guanosine-2',3'-cyclic monophosphate (PM4-B11)
Cytidine-2'-monophosphate (PM4-C8)
Cytidine-3'-monophosphate (PM4-C9)
Phospho-L-arginine (PM4-D4)
O-Phospho-D-serine (PM4-D5)
Omnilog units
Time (h)
Control
a ether (9a)
a ester (4a)
b ether (9b)

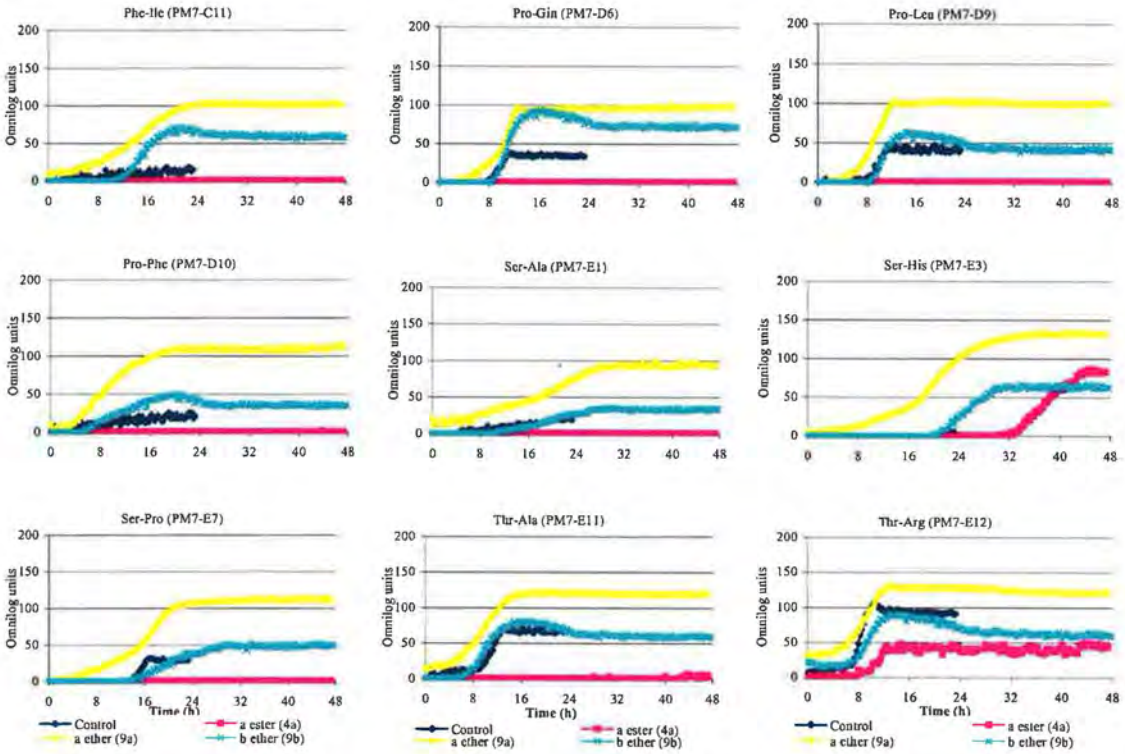

O-Phospho-D-Tyrosine (PM4-E1)
Phosphocreatine (PM4-E3)
Thiosulfate (PM4-F3)
Tetrathionate (PM4-F4)
Dithiophosphate (PM4-F6)
L-Cysteinyl-Glycine (PM4-F9)
N-Acetyl-L-Cysteine (PM4-G1)
S-Methyl-L-Cysteine (PM4-G2)
D-Methionine (PM4-G8)
Omnilog units
Time (h)
Control
a ether (9a)
a ester (4a)
b ether (9b)

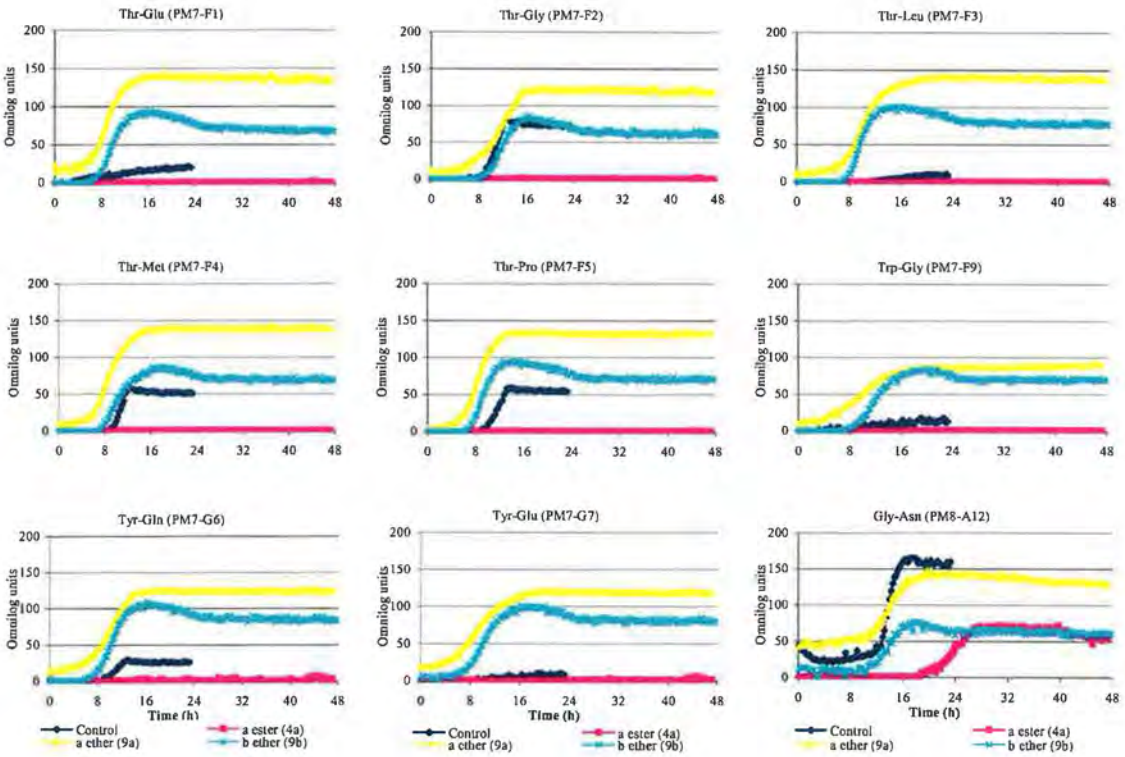

Thiourea (PM4-H2)
D,L-Lipoamide (PM4-H4)
2-Hydroxyethane sulfonic acid (PM4-H10)
Positive control: L-Glutamine (PM6-A2)
Ala-Arg (PM6-A4)
Ala-Asn (PM6-A5)
Ala-Glu (PM6-A6)
Ala-His (PM6-A8)
Ala-Leu (PM6-A9)
Omnilog units
Time (h)
Control
a ether (9a)
a ester (4a)
b ether (9b)

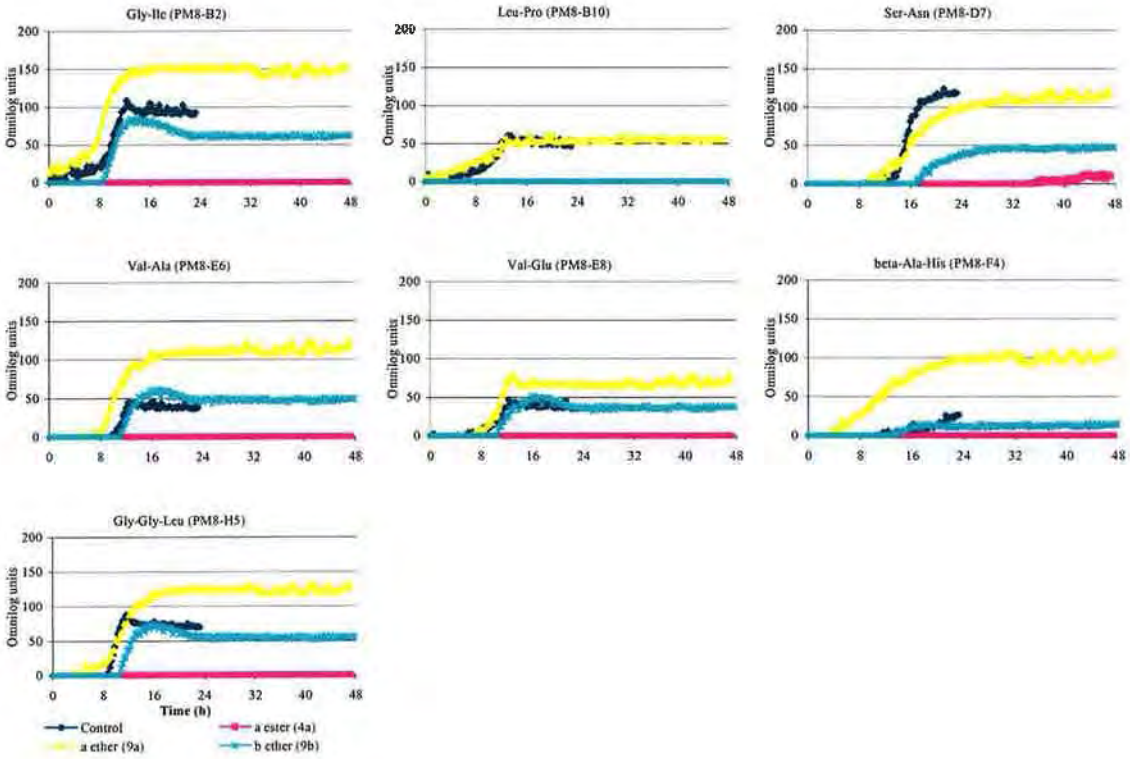

Ala-Thr (PM6-B2)
Arg-Gln (PM6-B8)
Arg-Glu (PM6-B9)
Arg-Val (PM6-C6)
Asn-Glu (PM6-C7)
Asp-Phe (PM6-D1)
Cys-Gly (PM6-D4)
Gln-Gly (PM6-D6)
Gly-Cis (PM6-E4)
Omnilog units
Time (h)
Control
a ether (9a)
a ester (4a)
b ether (9b)


Gly-leu (PM6-E7)
Gly-Thr (PM6-F1)
His-Gly (PM6-F6)
Ile-Ala (PM6-G3)
Ile-Gly (PM6-G6)
Ile-Val (PM6-H3)
Leu-Ala (PM6-H4)
Leu-Arg (PM6-H5)
Omnilog units
Time (h)
Control
a ester (4a)
a ether (9a)
b ether (9b)

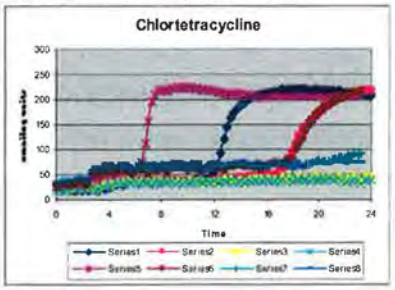

Positive control: L-Glutamine (PM7-A2)
Lys-Ala (PM7-A6)
Lys-Glu (PM7-A8)
Met-Gln (PM7-B9)
Lys-Thr (PM7-B3)
Met-Ile (PM7-C1)
Met-Phe (PM7-C5)
Met-pro (PM7-C6)
Met-Val (PM7-C8)
Omnilog units
Time (h)
Control
a ester (4a)
a ether (9a)
b ether (9b)

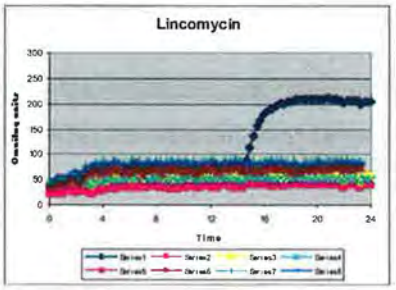

Phe-Ile (PM7-C11)
Pro-Gln (PM7-D6)
Pro-Leu (PM7-D9)
Pro-Phe (PM7-D10)
Ser-Ala (PM7-E1)
Ser-His (PM7-E3)
Ser-Pro (PM7-E7)
Thr-Ala (PM7-E11)
Thr-Arg (PM7-E12)
Omnilog units
Time (h)
Control
a ether (9a)
a ester (4a)
b ether (9b)

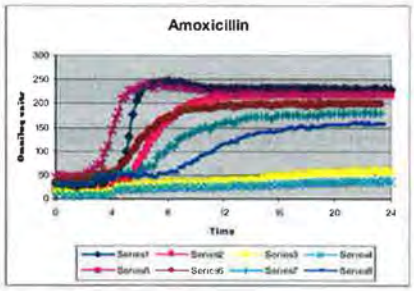

Thr-Glu (PM7-F1)
Thr-Gly (PM7-F2)
Thr-Leu (PM7-F3)
Thr-Met (PM7-F4)
Thr-Pro (PM7-F5)
Trp-Gly (PM7-F9)
Tyr-Gln (PM7-G6)
Tyr-Glu (PM7-G7)
Gly-Asn (PM8-A12)
Omnilog units
Time (h)
Control
a ether (9a)
a ester (4a)
b ether (9b)

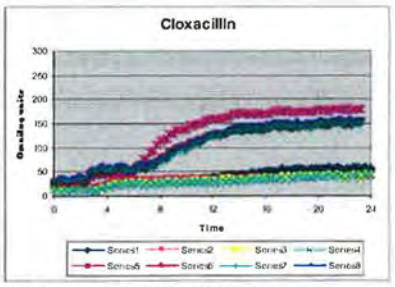

Gly-Ile (PM8-B2)
Leu-Pro (PM8-B10)
Ser-Asn (PM8-D7)
Val-Ala (PM8-E6)
Val-Glu (PM8-E8)
beta-Ala-His (PM8-F4)
Gly-Gly-Leu (PM8-H5)
Omnilog units
Time (h)
Control
a ester (4a)
a ether (9a)
b ether (9b)

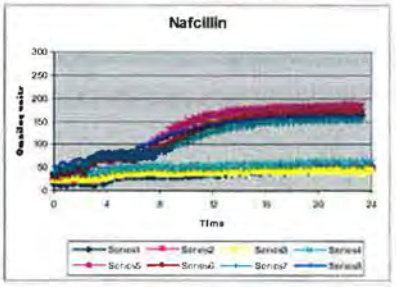
2.4.1 Alpha ester (4a) hits: PM11

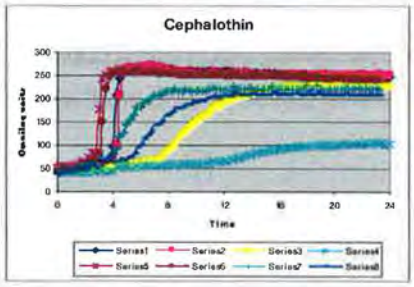




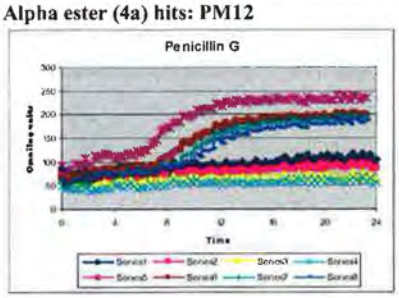


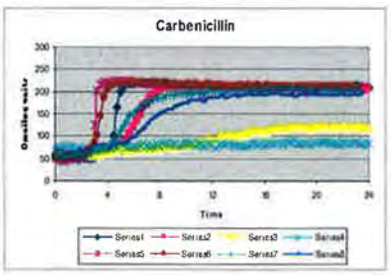


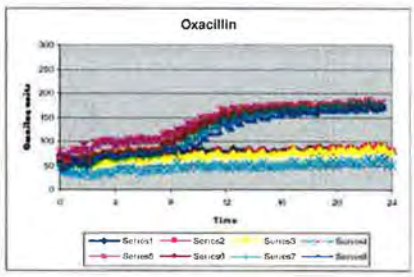


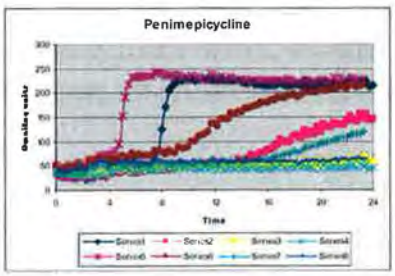


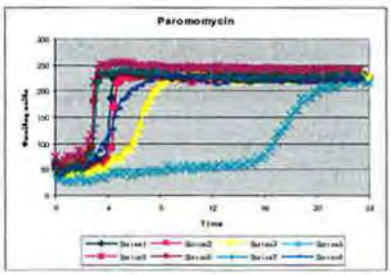
Alpha ester (4a) hits: PM12

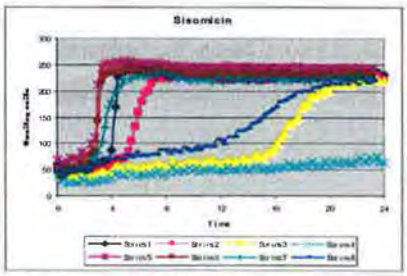




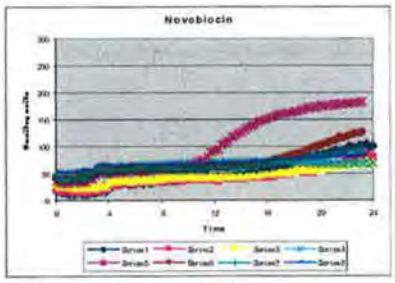


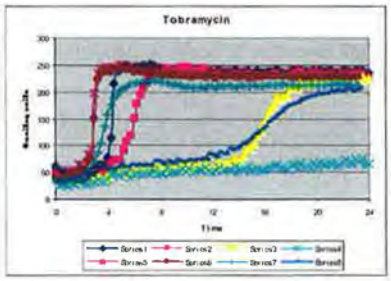


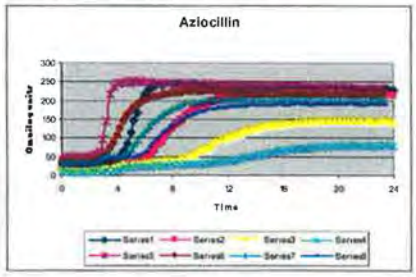




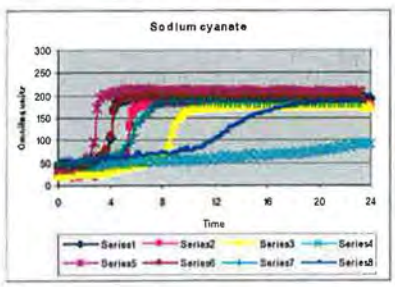
Alpha ester (4a) hits: PM13

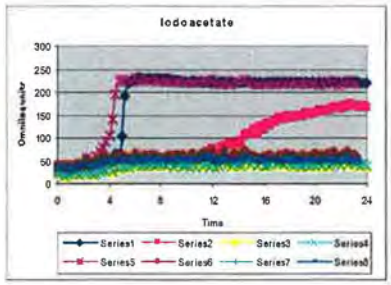


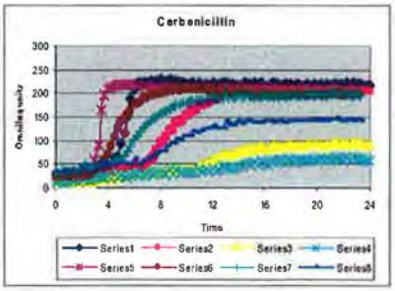




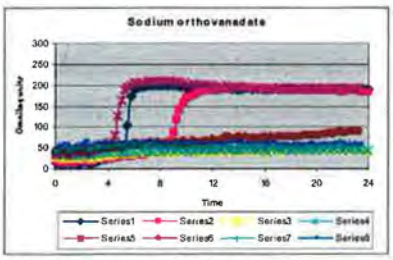
Alpha ester (4a) hits: PM14

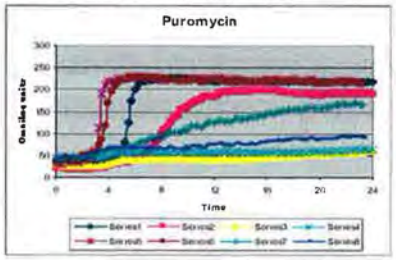


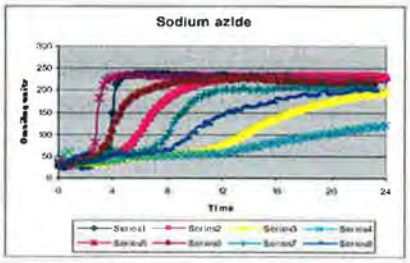




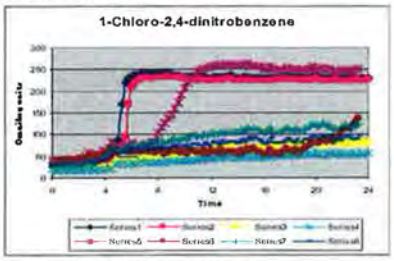
Alpha ester (4a) hits: PM15

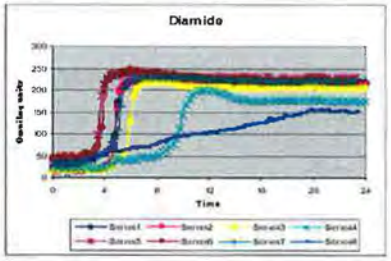


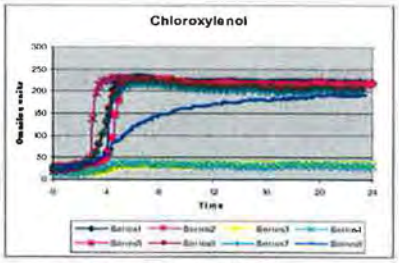




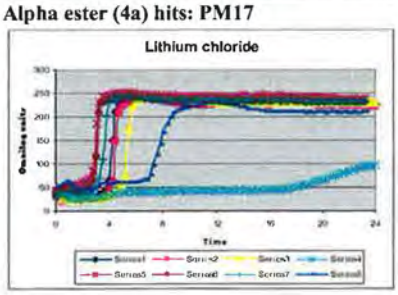
Alpha ester (4a) hits: PM16

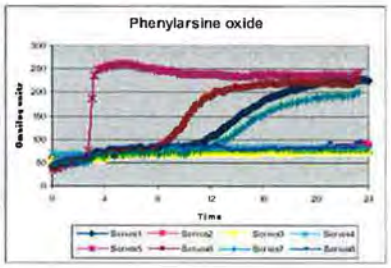




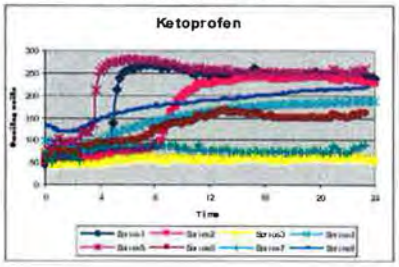


Alpha ester (4a) hits: PM17

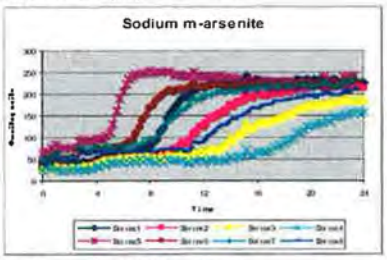


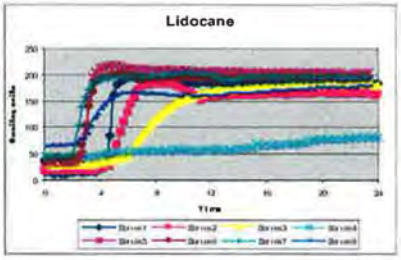


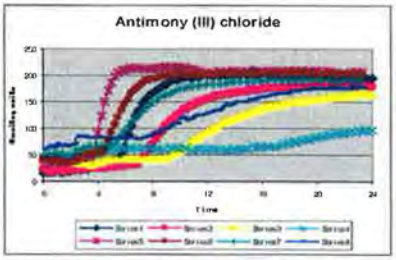
Alpha ester (4a) hits: PM18



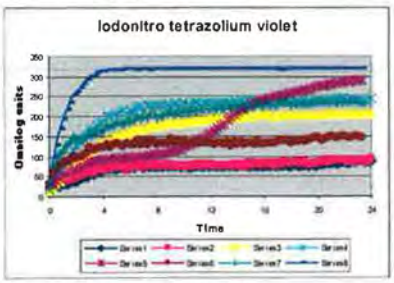
Alpha ester (4a) hits: PM18

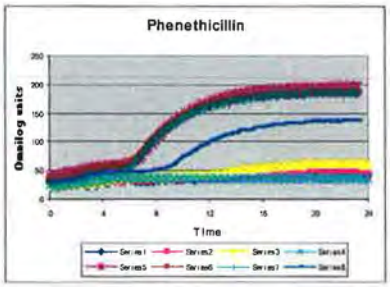


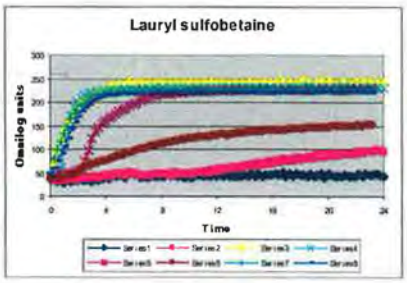




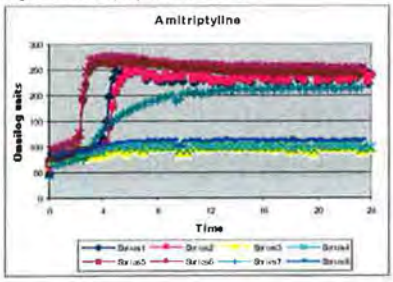
Alpha ester (4a) hits: PM19

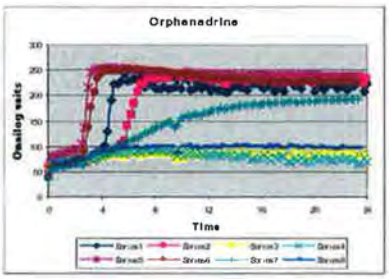


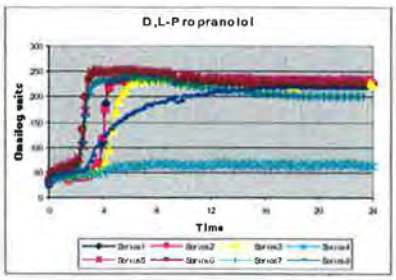




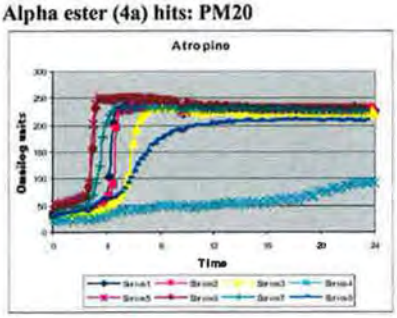
Alpha ester (4a) hits: PM20

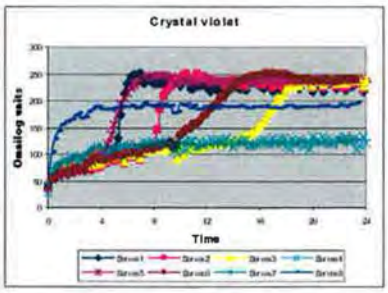


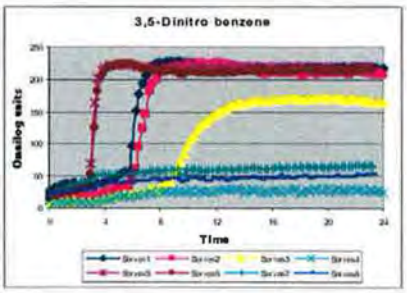


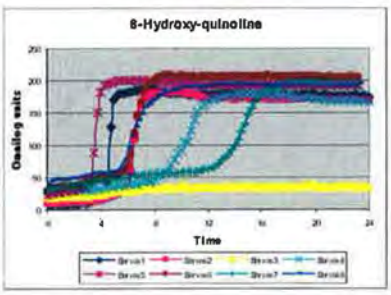


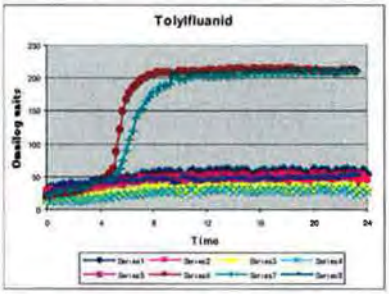
Alpha ester (4a) hits: PM20



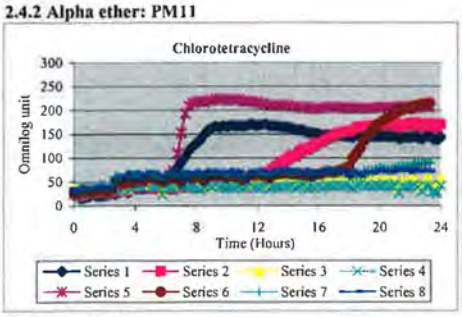




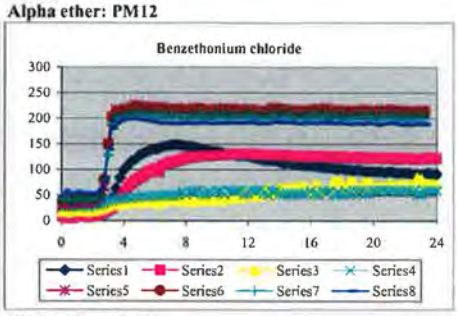


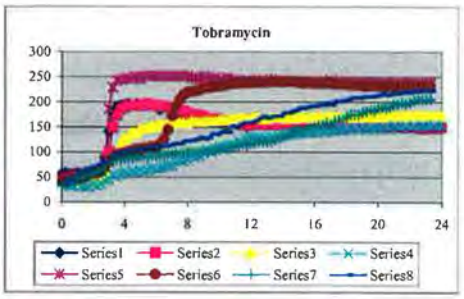


2.4.2 Alpha ether: PM11

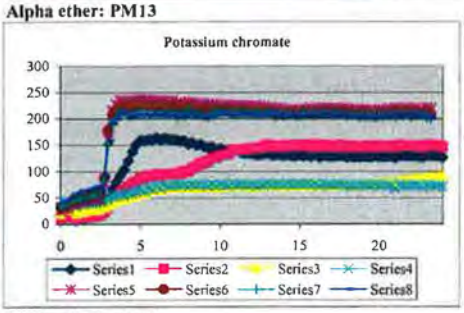


Alpha ether: PM12

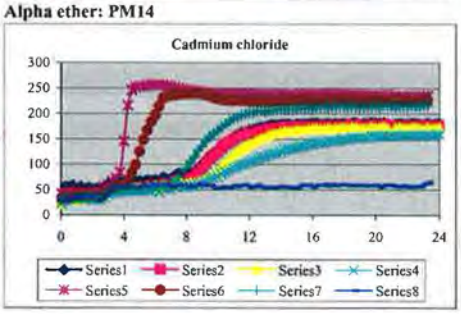


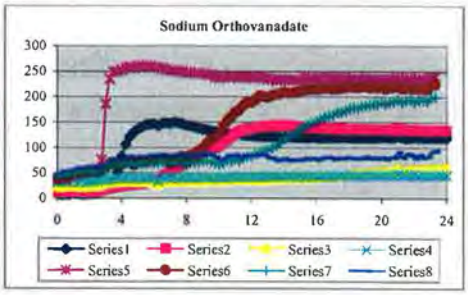


Alpha ether: PM13

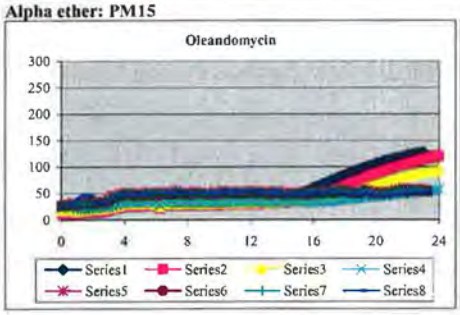


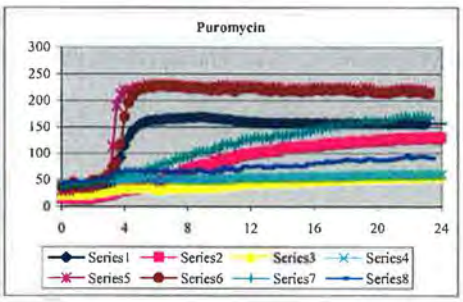
Alpha ether: PM14





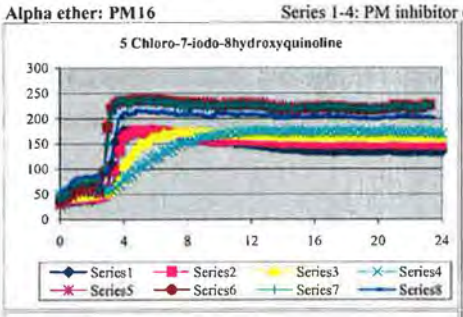
Alpha ether: PM15

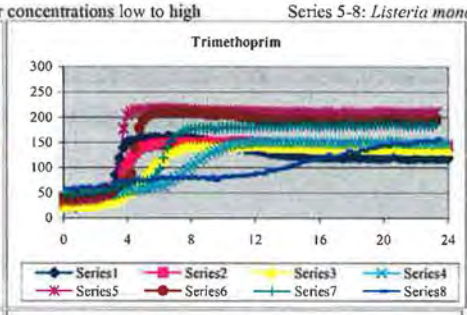


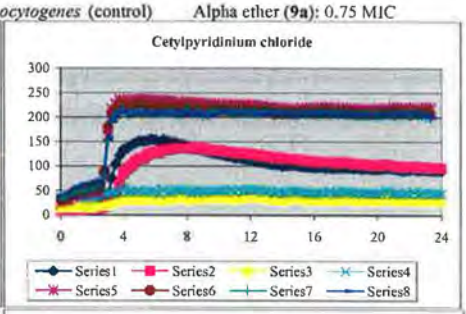


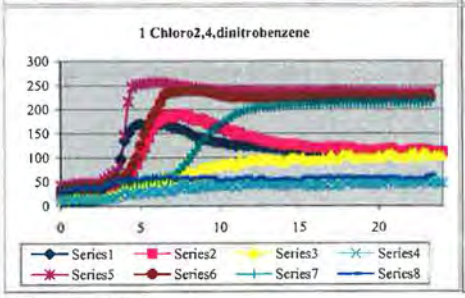
Series 1-4: PM inhibitor concentrations low to high
Series 5-8: *Listeria monocytogenes* (control)
Alpha ether (**9a**): 0.75 MIC

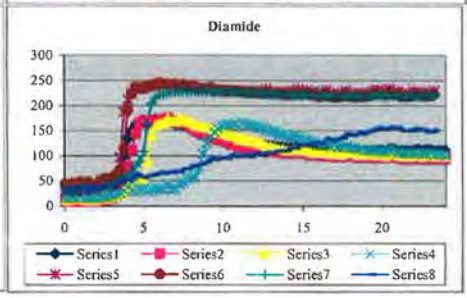
Alpha ether: PM16 Series 1-4: PM inhibitor concentrations low to high Series 5-8: *Listeria monocytogenes* (control) Alpha ether (**9a**): 0.75 MIC

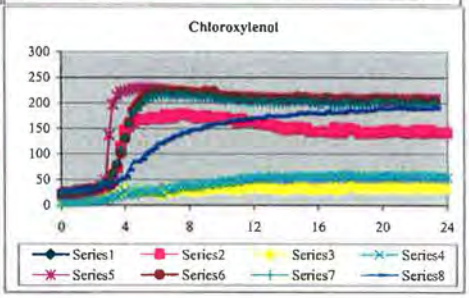




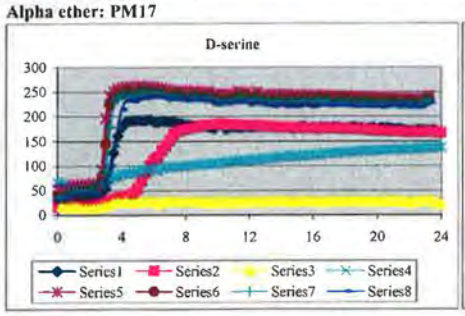


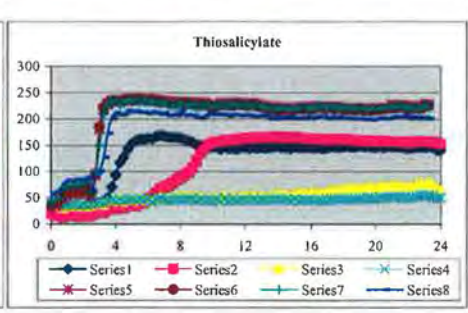


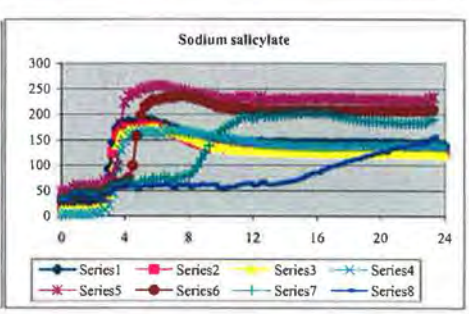




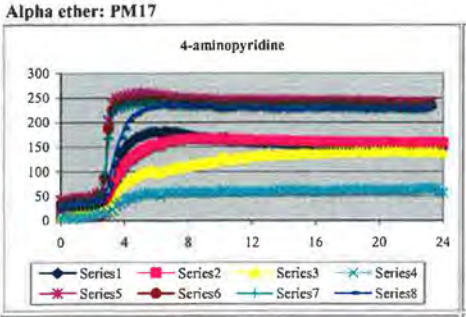
Alpha ether: PM17

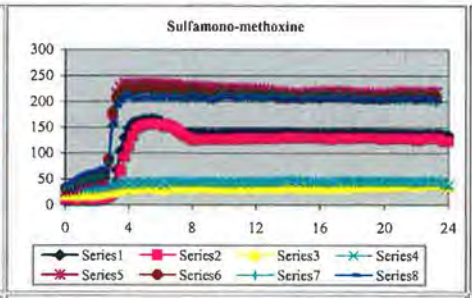


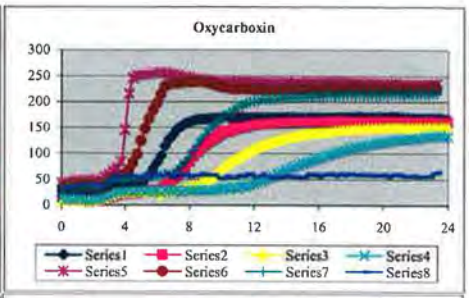


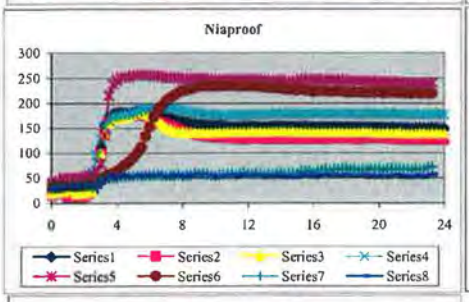


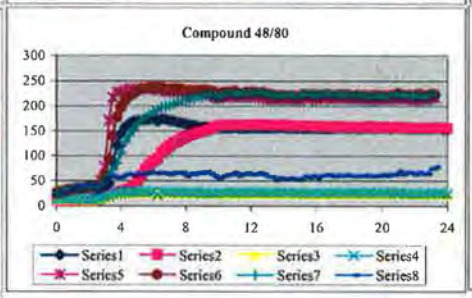
Alpha ether: PM17

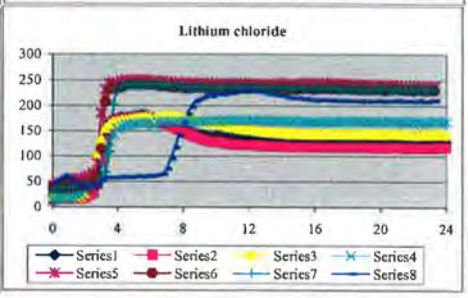


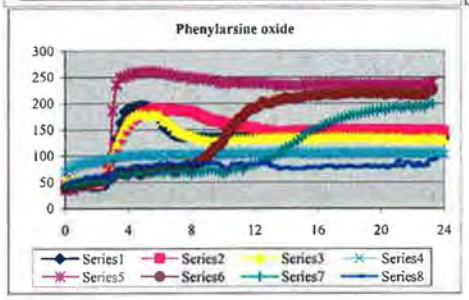






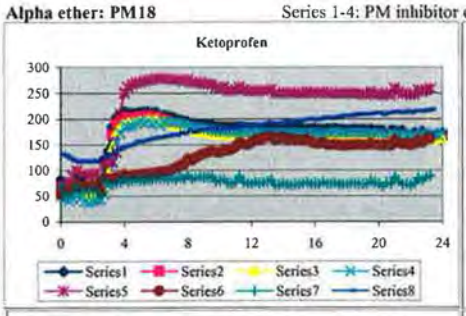


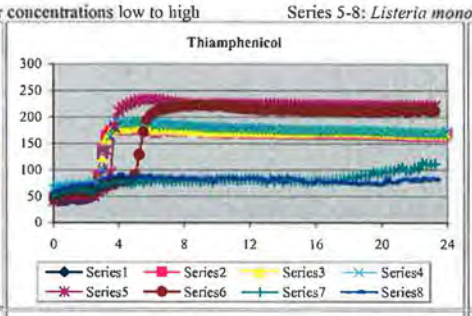


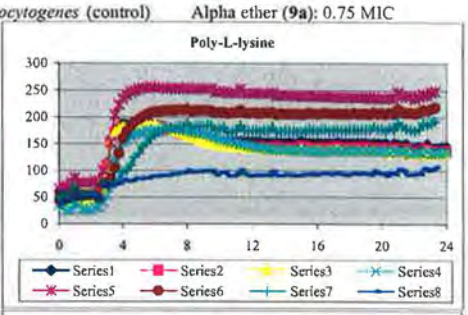


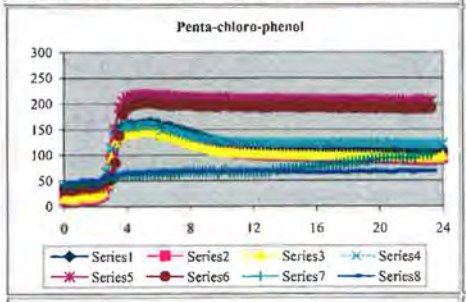
Series 1-4: PM inhibitor concentrations low to high
Series 5-8: *Listeria monocytogenes* (control)
Alpha ether (**9a**): 0.75 MIC

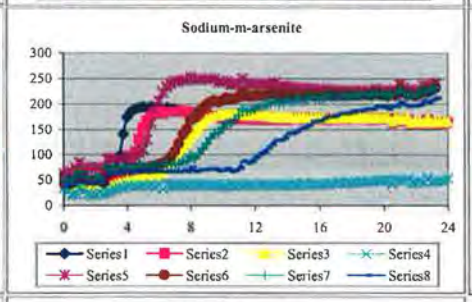
Alpha ether: PM18 Series 1-4: PM inhibitor concentrations low to high Series 5-8: *Listeria monocytogenes* (control) Alpha ether (**9a**): 0.75 MIC

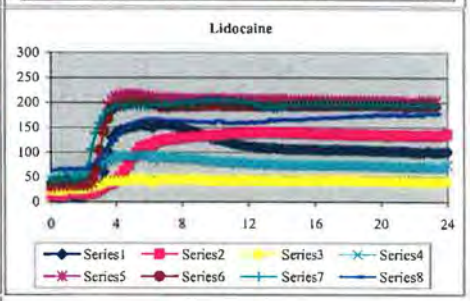


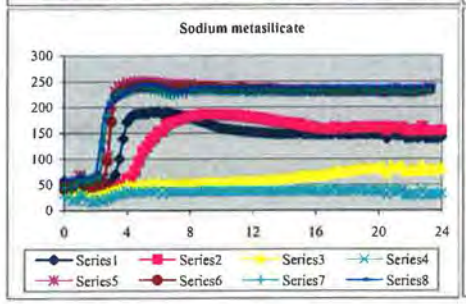


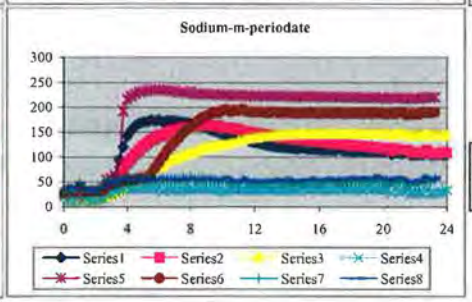






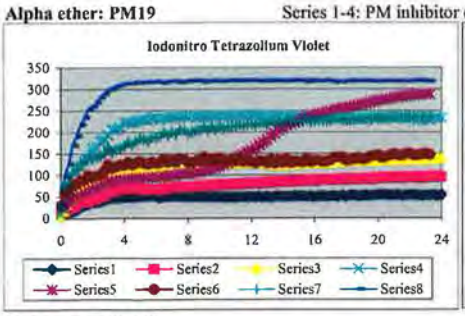


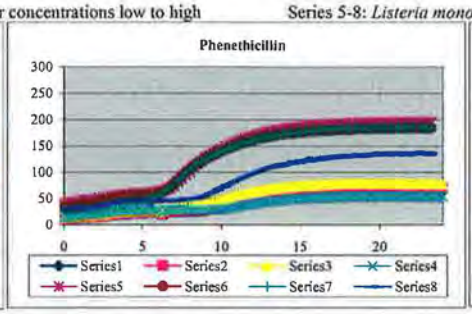


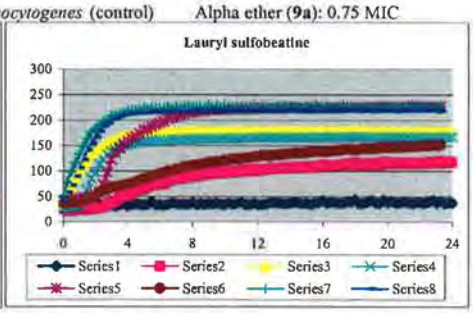


Series 1-4: PM inhibitor concentrations low to high
Series 5-8: *Listeria monocytogenes* (control)
Alpha ether (**9a**): 0.75 MIC

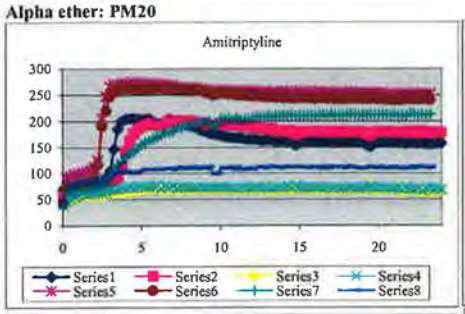
Alpha ether: PM19 Series 1-4: PM inhibitor concentrations low to high Series 5-8: *Listeria monocytogenes* (control) Alpha ether (**9a**): 0.75 MIC

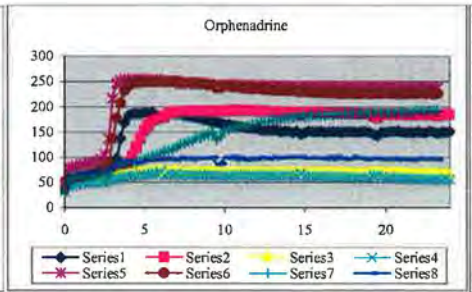


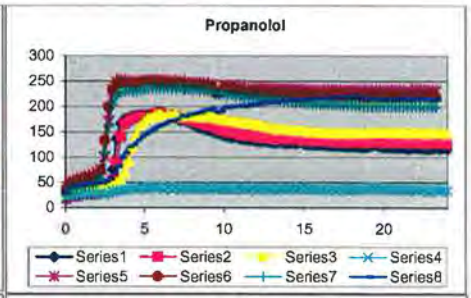


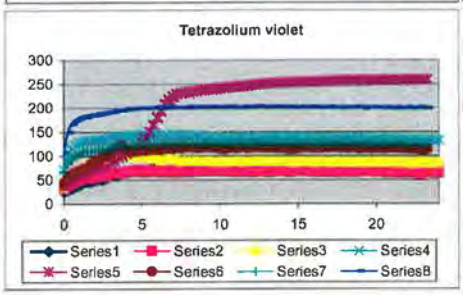


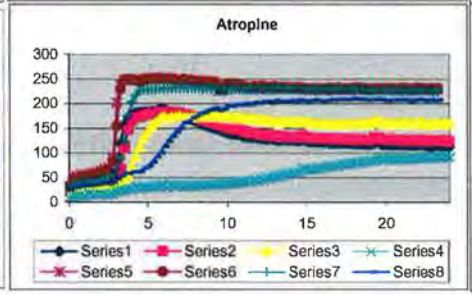
Alpha ether: PM20

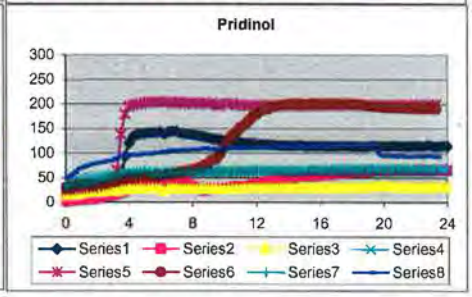


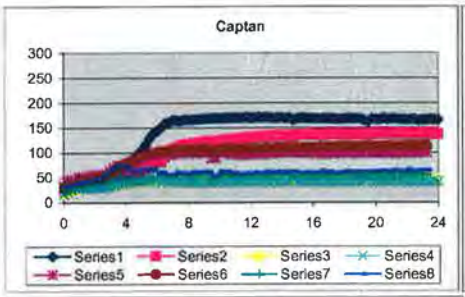


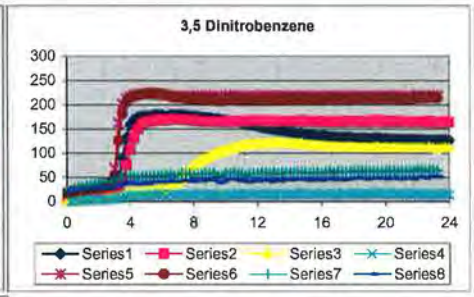


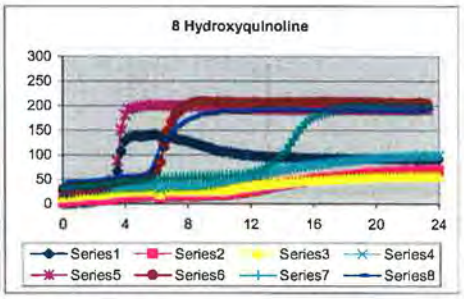


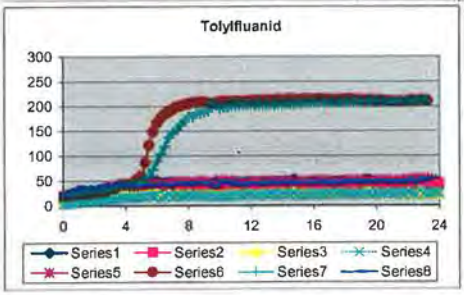






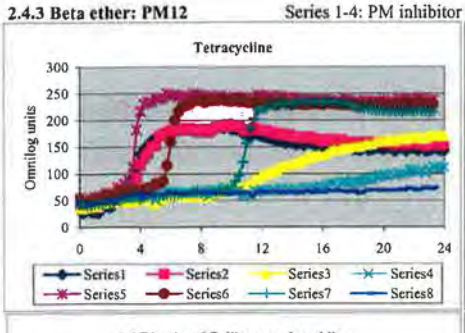


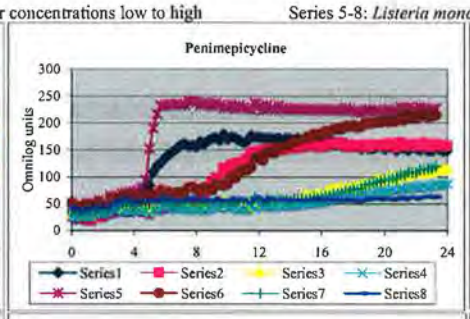


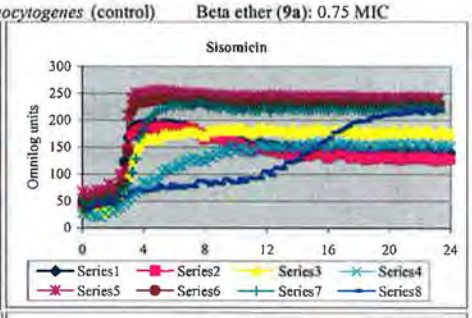


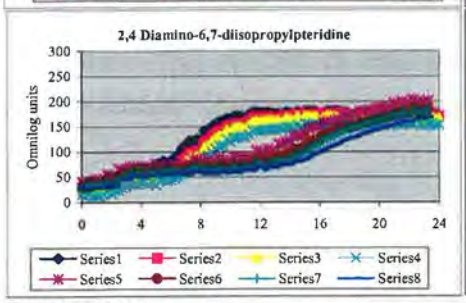
Series 1-4: PM inhibitor concentrations low to high
Series 5-8: *Listeria monocytogenes* (control)
Alpha ether (**9a**): 0.75 MIC

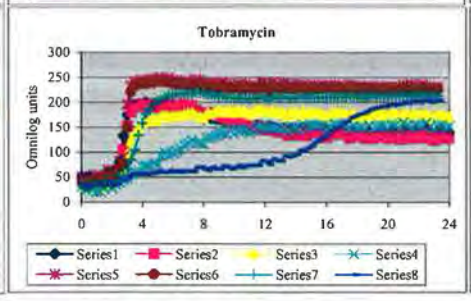
2.4.3 Beta ether: PM12 Series 1-4: PM inhibitor concentrations low to high Series 5-8: *Listeria monocytogenes* (control) Beta ether (**9a**): 0.75 MIC

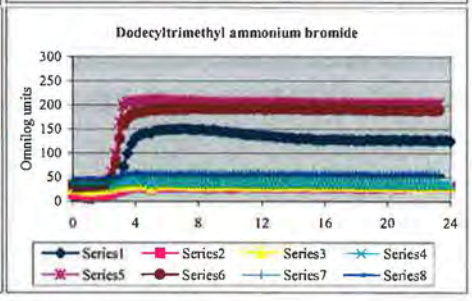




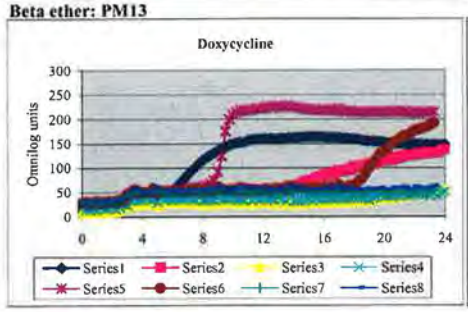


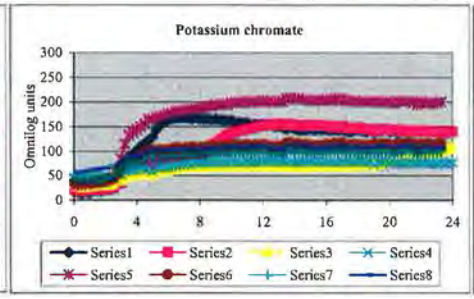


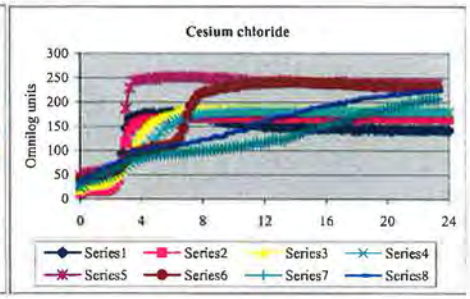




Beta ether: PM13

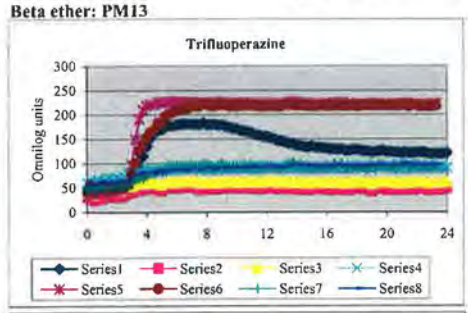




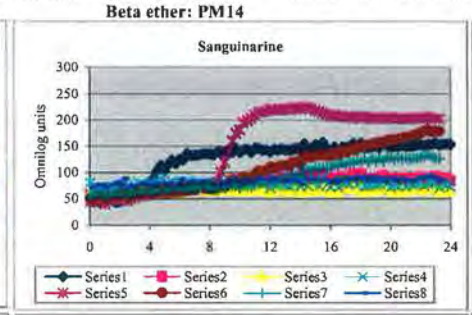


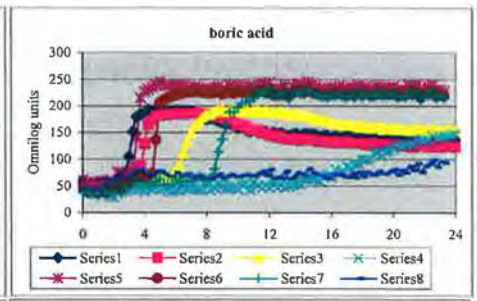
Series 1-4: PM inhibitor concentrations low to high Series 5-8: *Listeria monocytogenes* (control) Beta ether (**9a**): 0.75 MIC

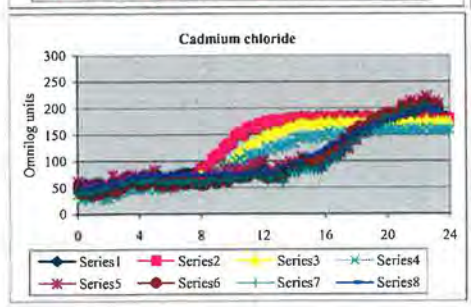
Beta ether: PM13

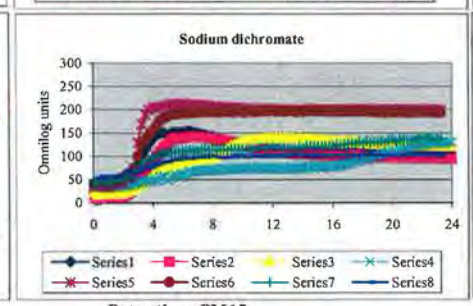


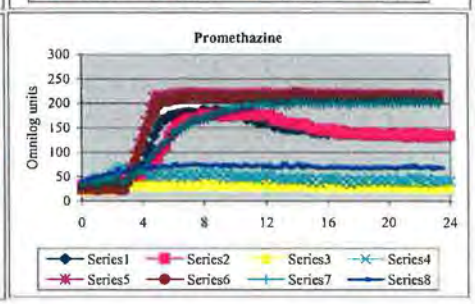
Beta ether: PM14



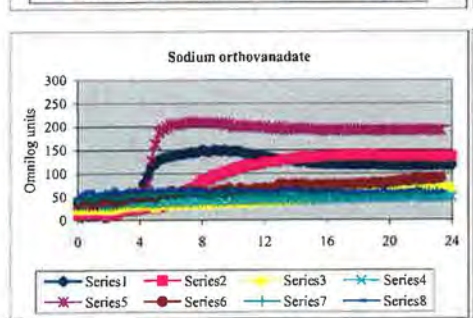


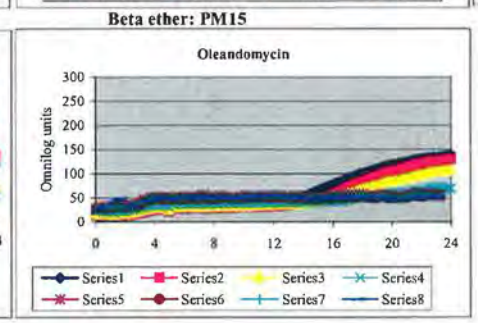




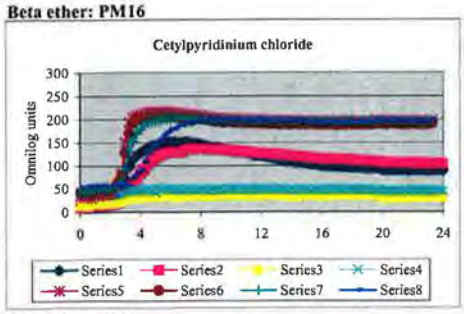


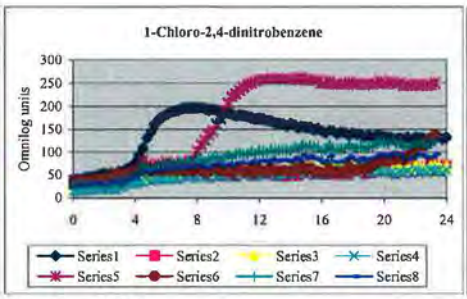
Beta ether: PM15



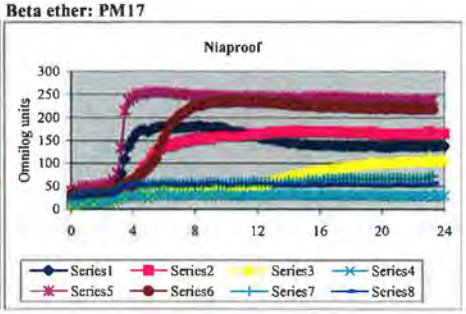


Beta ether: PM16

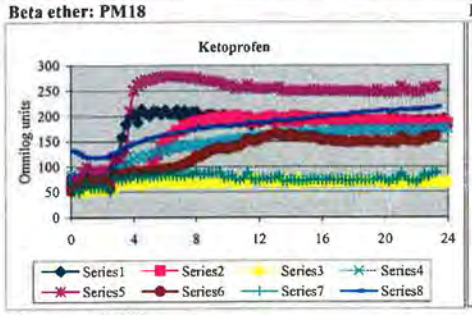




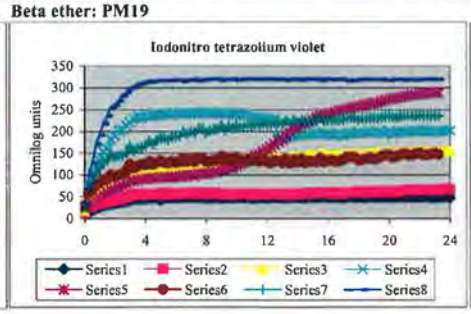
Beta ether: PM17

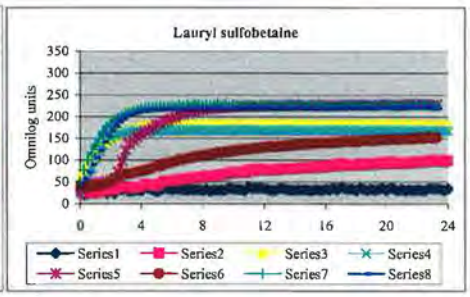


Beta ether: PM18

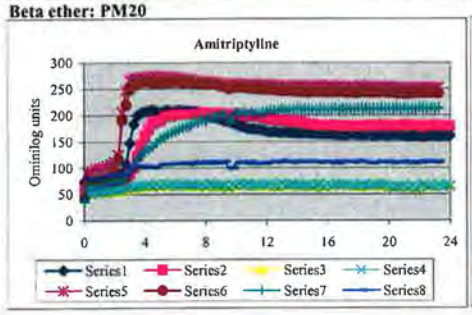


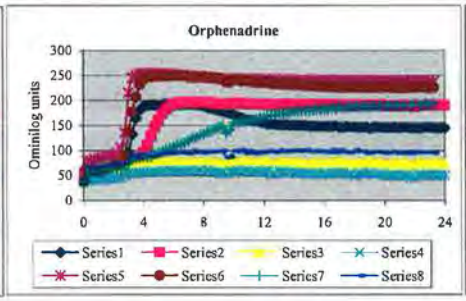
Beta ether: PM19

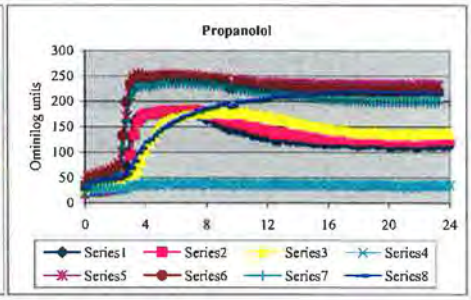




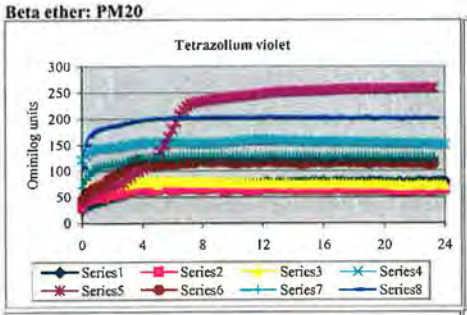
Beta ether: PM20

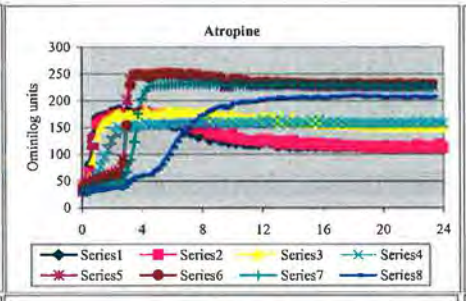


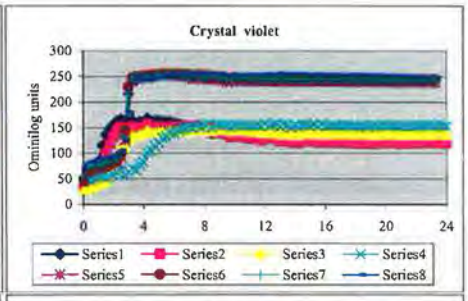


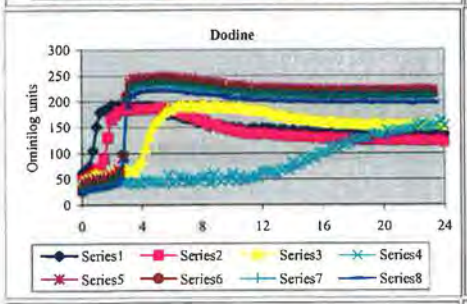


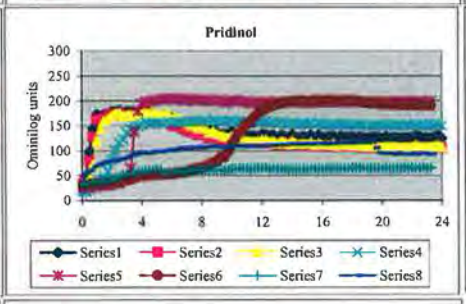
Beta ether: PM20

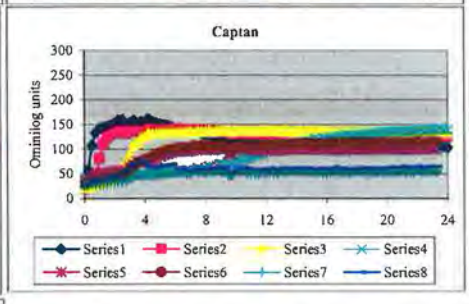


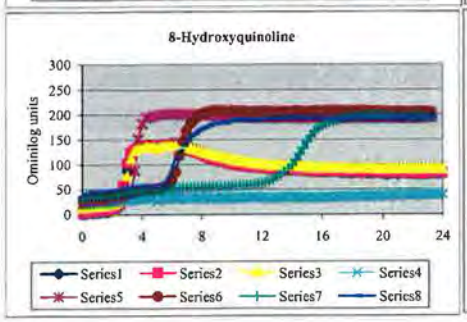


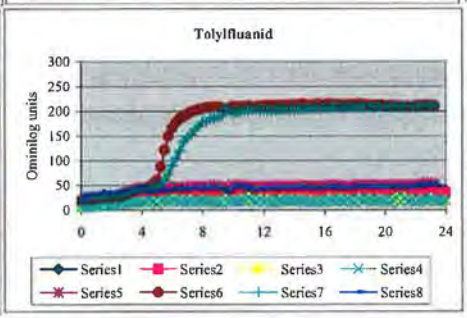












Beta ether (**9a**): 0.75 MIC
Series 1-4: PM inhibitor concentrations low to high
Series 5-8: *Listeria monocytogenes* (control)