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ANTIBACTERIAL PROPERTIES OF DITERPENES AND THEIR DERIVATIVES

A thesis presented in partial fulfilment of the requirements for the degree of
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ABSTRACT

Totarol is a diterpene isolated in large quantities from *P. totara* and a range of other plants, that has been shown to possess significant antibacterial activity against Gram positive bacteria. It has not been possible to unequivocally determine the mode of action by which this activity occurs. This research aimed to determine the mode of action of the diterpene and study a range of derivatives to elucidate a structure-function relationship for the diterpene to enable directional synthesis of future derivatives possessing increased activity and bioavailability.

The antibacterial activity of totarol and 29 derivatives was tested against *H. pylori* and *S. aureus*, two significant human pathogens, as representative Gram negative and Gram positive bacteria. Four compounds were found to possess significant activity against *S. aureus*, both MRSA and MSSA, although no significant activity was observed against *H. pylori*. The ability of the derivatives to potentiate the activity of existing β-lactam antibiotics such as methicillin was also investigated for MRSA and *E. coli*. Seven compounds including totarol were found to potentiate methicillin, one 256-fold, although no potentiation activity was exhibited against *E .coli*.

The incorporation of radiolabelled precursors was used to investigate the effect of totarol on the synthesis of three macromolecules, DNA, protein and peptidoglycan, in MRSA. No primary inhibition was detected, indicating that the mode of action of the diterpene was not inhibition of synthesis of any of these macromolecules.

The effect of totarol on the cellular respiration of MRSA was also investigated, showing 70 % inhibition of respiration at MIC levels, and complete inhibition of respiration at five times that concentration. It was therefore concluded that this was the most likely primary antibacterial effect of the compound.

The effect of totarol on the production of PBP 2a, an important protein in the β-lactam resistance mechanism of MRSA, was also investigated using a novel, non-radioactive labelling procedure to detect the protein. However, although a variety of

strategies were employed to detect the protein, none were successful, and the experiment set aside until the arrival of anti-PBP 2a antibody for use in another strategy.

Future work on this project that could be undertaken includes determination of the effect of the derivatives on cellular respiration under potentiation conditions, determination of the component(s) of the respiratory chain affected by totarol, and the investigation of the effect of the diterpene on PBP 2a production and function using antibody to detect the protein.

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1 INTRODUCTION

1.1 Diterpenes and Their Origins

Terpenes are a group of chemical compounds widely distributed throughout nature, mainly in plants as constituents of essential oils (Kirste, 1998). The building block of these compounds is isoprene and the name of the compound determined by the number of isoprene units contained within it; therefore diterpenes have two such molecules (Kirste, 1998). Terpene-like molecules include many commonly encountered compounds such as camphor and menthol (Kirste, 1998). Many such compounds exist in nature, one of which, totarol, is the focus of this research.

Totarol was first described in the hardwood tree, *Podocarpus totara,* commonly called totara and indigenous to New Zealand, by Easterfield in 1910 (reviewed by Bendall and Cambie, 1995). Easterfield reported that crystals of the compound could be seen on totara boards several hours after planing. Since that time, totarol has been found in a large number of plants, ranging from other species of Podocarps such as *P. nagi*, to species of Juniper and the herb rosemary (Bendall and Cambie, 1995). However, the most abundant source of the diterpene remains the heartwood of *P. totara* from which it can be obtained in a yield of 5 to 8 % of the dry weight of the timber (Bendall and Cambie, 1995). Totarol is in fact the major chemical constituent in the hexane extract of the heartwood of *P. totara* (G. Evans *et al.*, 1998). The genus *Podocarpus* is well known as a rich source of diterpenoids and of this genus *P. nagi* is the best studied (Ying and Kubo, 1991). This species also provides a source of totarol as well as other diterpenes and other compounds possessing antimicrobial activity such as anacardic acid.

Biochemical and microbiological studies have been conducted on many of these compounds and a variety, including totarol, anacardic acid and pisiferic acid have been found to possess intrinsic antimicrobial activity (Muroi and Kubo, 1994). Totarol and anacardic acid have been found to possess potent activity against Gram

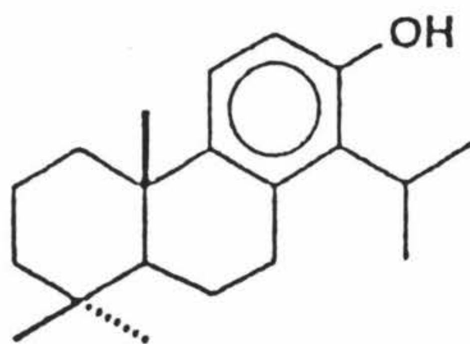
positive bacteria, in particular *Staphylococcus aureus* (Muroi and Kubo, 1996). This makes them attractive as potential chemotherapeutic agents, as very few plant-derived compounds are active against *S. aureus* (Muroi and Kubo, 1996), a bacterium which is frequently multi-resistant to many common antibiotics.

Although totarol is described as a diterpenoid, its structure (Figure 1.1) does not actually follow the isoprene rule, having an isopropyl group at the "wrong carbon". However this contributes to the unique chemistry of the compound which includes the fact that it is a useful compound for semi-synthetic work (Bendall and Cambie, 1995). This is important in relation to this work which will investigate the antimicrobial activity of compounds derived from totarol.

1.2 Natural Product Antibiotics

Currently many antimicrobial agents in use are of natural origin. Many of the original antibiotics used were produced by micro-organisms, in particular Actinomycete bacteria, as secondary metabolites (Monaghan and Tkacz, 1990). However the use of plants and plant extracts in human medicine is recorded throughout history, and today plant-derived compounds remain a significant proportion of pharmaceuticals employed clinically (Monaghan and Tkacz, 1990). For medicinal plants and herbs, the plants tested were often picked solely on the basis of previous use in history for such purposes, for example tea-tree oil in the treatment of burns (Foagali *et al.*, 1997). However, with current knowledge and understanding of cellular processes, it has become possible to target enzymes or receptors for chemotherapeutic agents with certain desirable modes of action, and thereby specifically screen compounds for this activity (Monaghan and Tkacz, 1990). It is by the more selective screening procedures used today that many current antibiotics

Figure 1.1 The structure of totarol


OH

have been found to possess unexpected activities. An example of this is Ascomycin, a compound reported in 1962 as an antifungal compound, which has recently been found also to be active in lowering cholesterol production in humans (Monaghan and Tkacz, 1990).

Recently several novel antimicrobial agents were isolated in plants and are in the process of being tested. Products such as various macrocarpals from *Eucalyptus macrocarpa* and new diterpenoids from the Lamiaceae group of plants have all recently been described, and may have applications in the future (Behroozi *et al.*, 1996; Dellar *et al.*, 1996; McDaniel *et al.*, 1995; Yamakoshi *et al.*, 1992). The macrocarpal derivatives are promising as they possess activity against Gram positive bacteria including *S. aureus* (Yamakoshi *et al.*, 1992). Other natural antibiotic agents are also derived from different sources such as leinamycin from *Steptomyces* or mutastein from *Aspergillus terreus* the latter of which inhibits the synthesis of an insoluble glucan by *Streptococcus mutans*, a bacterial species associated with the formation of dental plaque (Behroozi *et al.*, 1996; Monaghan and Tkacz, 1990). This is reportedly being used commercially as a prophylactic agent for tooth decay (Monaghan and Tkacz, 1990).

However, plants remain an important source of novel antibiotic agents. The genus *Podocarpus* has provided a good source of antimicrobial agents, including diterpenes such as totarol and pisiferic acid and other products such as anacardic acid, all of which are active against Gram positive bacteria (Muroi and Kubo, 1994; Kubo *et al.*, 1992; Kobayashi *et al.*, 1988;) and have been investigated to varying degrees in order to determine their potential as future chemotherapeutic agents.

With increasing knowledge and understanding of how these natural products work it may be possible to synthesise related products with similar or improved antimicrobial characteristics. This has been investigated in the case of polyketides, a large family of natural products with a wide range of biological activities (McDaniel *et al.*, 1995). Some of the more well-known polyketides include the antibiotics erythromycin and

tetracycline, immunosuppressants, anticancer compounds and antiparasitics. All of these are produced by Actinomycete bacteria and have a common mode of synthesis including polyketide synthase enzymes and repeated condensation cycles (McDaniel *et al.*, 1995).

McDaniel *et al.* (1995) discussed the rationale behind the design of novel polyketides with specific functions; such design of antimicrobial agents could potentially be applied to many other classes of compounds, for example diterpenes or other plant products, if their synthesis pathways were elucidated.

Natural products are invaluable for defining new drug targets; the determination of the mode of action of taxol (paclitaxel) showed that microtubule stabilisation was a viable approach to cytotoxicity and the importance of various toxins in revealing many classes of ion channels are two of many recent examples that could be suggested (Petsko, 1996).

1.3 Design of Novel Antibiotic Agents

By the mid-1980's more than 6000 antibiotics had been discovered (Omura, 1986). However, the increasing rates of antibiotic-resistant bacteria, in particular Gram positive bacteria such as Staphylococci, Enterococci and Pneumococci have made it important to have new antibiotics on hand, which may have novel modes of action or attack newly recognised targets in the bacteria. Before the appearance of such resistant bacteria, in particular the staphylococci where methicillin-resistance is often correlated with multi-drug resistance, it was widely believed that there were enough antibiotics in use for the treatment of all bacteria and that as a result significant investment in antibiotic discovery and development was not warranted (Ford *et al.,* 1997). To compound the problem, during the past three decades, the emphasis had been placed on newly discovered antibiotics with improved activity against Gram negative bacteria, with the consequence being a decrease in the number of antibiotics possessing good activity against Gram positives (Ford *et al.*, 1997, Baquero). This

has helped contribute to the emergence of antibiotic-resistant Gram positive bacteria as a serious therapeutic problem.

In order to combat these bacteria it became obvious that there was a need for novel chemotherapeutics. Vancomycin is the antibiotic of choice for treating methicillin-resistant *Staphylococcus aureus* (MRSA) infections due to their propensity for multiple resistance. However, the emergence of vancomycin-resistant Enterococci and the subsequent discovery of a vancomycin-resistant strain of MRSA (Hiramatsu, 1997; Hiramatsu, 1997) has increased the pressure for alternative antibiotics. There is now a very real need for the development of novel antibiotics to treat these bacteria.

As mentioned above, the majority of antibiotics come from natural sources, either plants or micro-organisms (Knüsel and Nüesch, 1990). However, it is difficult to isolate novel antibiotics from these sources for several reasons. The synthesis of such natural products is very complex (Verdine, 1996), and there are often many steps and enzymes involved in the biosynthetic process, making synthesis of the compounds and their analogues very difficult (Petsko, 1996). Organisms that are similar genetically will often produce compounds that are chemically similar (Verdine, 1996) so it is necessary to sample a very broad array of micro-organisms, from the most diverse environments in order to obtain the broadest range of structures and compounds. Currently niches such as coral reefs, deep-sea hydrothermal vents and tropical rainforests are being scoured for novel antibiotics or lead compounds that could be developed further (Verdine, 1996). The effect the product will have on the cells of the animal that is to be treated must also be considered. Compounds that are toxic for other micro-organisms can also be toxic for humans. Sometimes it is not possible to predict the chemical reactions micro-organisms will utilise. For example the isomerisation of isobutyrate to *n*-butyrate in the biosynthesis of leucomycin and tylosin was a reaction that had never been seen before in organic chemistry (Omura, 1986). Biologically derived compounds are the most chemically diverse known (Petsko, 1996).

The complexity of naturally occurring antibiotics is one hurdle to overcome in the production of new ones. The polyketide class of compounds includes a large number of antibiotics such as tetracyclines, erythromycin and other molecules, including immunosuppressants and anticancer compounds, many of which are produced by the Actinomycetes (Knüsel and Nüesch, 1990; McDaniel *et al.*, 1995). They are synthesised by enzymes of very large molecular weight called polyketide synthases in long and complicated cycles of condensation (Verdine, 1996). However, a target for the development of novel partially synthetic polyketides exists as the enzymes are ordered sequentially in operons, the sequence of which relate directly to the domains added to the molecules (Verdine, 1996). As a result it was postulated that it may be possible to create novel polyketides by designing new polyketide synthases or by the construction of combinatorial libraries of genetically engineered polyketide synthases (McDaniel *et al.*, 1995). This concept was fostered by the early success in producing the 'hybrid' polyketides mederrhodin A and dihydrogranatirhodin (McDaniel *et al.*, 1995).

In most cases however, not enough is known about the biosynthetic pathways of such compounds and researchers must resort to other methods to try and develop novel antibiotic agents. In the past, these efforts relied heavily on screening procedures. However, these require prior knowledge on the organisms being tested as it is necessary to be able to isolate and cultivate the micro-organisms and also to be able assay a small quantity of the substance easily and quickly (Omura, 1986). If such information is available, different screening procedures can be applied for the different targets of the antibiotics (Omura, 1986). Manipulations of fermentation conditions can also be beneficial as this can result in greater antibiotic production or it may induce non-producing strains to start producing (Omura, 1986).

However, as the demand for novel antibiotics increases, along with the pressure to produce them quickly and cheaply (Verdine, 1996), newer methods are being developed in order to not only test micro-organisms faster, but also to synthesise new, related compounds more easily and quickly. Ways in which this can be accomplished include high throughput screening, structure-based drug design and

combinatorial chemistry (Petsko, 1996). High throughput screening generally provides a starting point for drug discovery, as a precise determination of a macromolecular target is not necessary (Broach and Thorner, 1996) and can identify possible lead compounds that can be further studied. Often the emphasis is placed on finding lead compounds that can be manipulated, although the need for low toxicity, good pharmacokinetics, oral bioavailability and other factors can limit the efficacy of such compounds (Petsko, 1996).

Combinatorial chemistry is a synthesis strategy based on the production of all possible combinations of a basic set of modular components (reviewed by Hogan, 1996). Most combinatorial compound arrays are made by the random attachment of various functional groups to a central core. Often the scaffold has been chosen for ease of synthesis or low toxicity, although sometimes it can confer biological activity itself (Hogan, 1996). Combinatorial chemistry can provide much more scope with the potential development of the production of large sets of related molecules (Hogan, 1996) and is a process related to the way in which micro-organisms develop novel compounds; all biopolymers are formed in this manner (Petsko, 1996).

The development of entirely synthetic compounds can be effective. An example of this is the recent development of oxazolidinones, potent inhibitors of protein synthesis in Gram positive bacteria and entirely synthetically manufactured (Ford, *et al.*, 1997). Oxazolidinones are also important because their inhibition of protein synthesis occurs at a stage not affected by any marketed antibiotics (Ford, *et al.*, 1997). Cerulenin, isolated from *Cephalosporium ceaerulens* was found to be the first specific inhibitor of fatty acid biosynthesis (Omura, 1986) which is thought to contribute to its broad antimicrobial activity. Exploitation of newly isolated cellular targets is another way in which to develop novel antibiotics.

Rational derivatisation is the way in which the derivatives in this research were developed. The derivatisation programme develops new derivatives by comparing

the structure of existing diterpenes with antibacterial activity to determine structural features necessary for the antibacterial activity of the compound, such as the carboxyl group in pisiferic acid (Kobayashi *et al.*, 1989) or the phenolic group in totarol (Evans *et al.*, 1998) and derivatives retaining those structural features are created. This type of antibiotic development strategy was previously used in the development of semi-synthetic β-lactam antibiotics such as methicillin, ampicillin and oxacillin. These compounds are all derived from 6-amino penicillanic acid, a compound that is easily obtainable by controlled fermentation of *Streptomyces* or the deacylation of penicillin G (Synthesis of Penicillin, Cephalosporin C and Analogues, M. S. Manhas and A. K. Bose, 1969, Marcel Dekker, New York).

Through many years of research into semi-synthetic β-lactams, important structural features have been identified, the manipulation of which can have dramatic effects on the antibacterial activity of the resulting compound. The fused β-lactam-thiazolidine ring system is essential for the antibacterial activity of penicillins (Manhas and Bose, 1969). However, side chains of this central ring can be altered, the nature of the alteration conferring a level of activity on the compound. The acylamino side chain is important both for antibiotic activity and is also an important factor in the susceptibility of penicillins to nucleophilic and electrophilic attack. The addition of an amino group in the side chain has been found be important for the effectiveness of penicillins and activity is reduced if this group is substituted (Manhas and Bose, 1969.).

It has also been found that sterically crowded side chains confer resistance to degradation by penicillinase. Antibiotics with side chains of this type include methicillin (Doyle *et al.*, 1962). Alterations to side chains have also been found to decrease the activity of penicillins; a phenyl substituent at C5 will remove all activity (Sheehan and Laubach, 1951), while it was found early in semisynthetic β-lactam research that replacement of the methyl groups at C2 with hydrogen or other methyl groups result in lower activity.

Therefore, by using the above knowledge gained over the past fifty years it is possible to synthesise semi-synthetic β-lactams with the desired characteristics of lower excretion rate and toxicity and higher activity against a range of organisms.

1.4 Antibacterial Activity of Diterpenes

Diterpenes include a large number of compounds, several of which have been found to possess antibacterial activity. There are many advantages to the use of diterpenes as antibacterial agents; totarol in particular is produced in large quantities, such that crystals can be recovered from the walls of kilns used to dry totara boards (Bendall and Cambie, 1995). The antimicrobial effect of the compound has been seen for many years; totara timber is known to be very resistant to rotting due to the strong antibacterial effect of totarol, mostly against Gram positive bacteria although it is only recently that the activity of totarol has been studied (Bendall and Cambie, 1995).

1.4.1 Importance of Structure on Antibacterial Activity

Prior to the commencement of this work, a number of studies had been conducted on diterpenes to test their antibacterial activity, against a variety of bacteria, both Gram positive and -negative. Diterpenes tested include totarol and pisiferic acid (Haraguchi *et al.*, 1996; Muroi and Kubo, 1994; Kubo *et al.*, 1992; Kobayashi *et al.*, 1989; Kobayashi *et al.*, 1988). It was reported that the structure of the diterpenes had a profound effect on their activity. In the case of pisiferic acid it was found that carboxyl and hydroxyl groups were important for its activity (Kobayashi *et al.*, 1988). In that study it was found that only derivatives of pisiferic acid containing the C(10) carboxyl group had any antibacterial activity. A similar trend was observed in a study by Evans *et al.* (1998), involving the synthesis of 20 systematically modified analogues of totarol. In this study it was found that the activity of the compounds appeared to be dependant on the presence of a phenolic group; only those analogues possessing the group retained potent activity against Gram positive bacteria. In a study in 1992 by Kubo *et al.,* it was found that derivatives of totarol with an

additional hydroxy group at the 3-position had decreased activity, and that different levels of oxidation at the 4β-methyl group caused dramatic decreases in activity. If the methyl group is oxidised the biological activity of the compound was completely lost up to concentrations of 400 μg/ml.

1.4.2 Effect of Lipophilicity on Antibacterial Activity of Diterpenes

Lipophilicity has also been found to be an important factor in the activity of diterpenes. Kobayashi *et al* (1988) reported that the activity of pisiferic acid was affected by the lipophilicity of compounds; the more lipophilic compounds were trapped in the cell envelope lipid phase of Gram negative bacteria but could pass through the Gram positive cell wall. As a result the more lipophilic compounds were active against Gram positive bacteria while the less lipophilic compounds possessed more activity against Gram negative bacteria (Kobayashi *et al*., 1988). Indeed, the unionised form of totarol is very lipophilic (Evans *et al*., 1998) which could account for its strong activity against Gram positive bacteria.

The diterpenes discussed above are antibacterial for Gram positive bacteria only, with the exception of pisiferic acid which also possesses some activity against Gram negative bacteria, although this appears to be non-specific (Kobayashi *et al*., 1988). Totarol has been found to possess no activity against Gram negative bacteria when tested at concentrations up to 400 μg/ml, although it was found to be potently active against all the Gram positive bacteria used in a study by Kubo *et al*. (1992). However, the activity of diterpenes against Gram positive bacteria is a very useful property, particularly those that are active against *S. aureus*. Although antibacterial activity against Gram positive bacteria is common among phytochemicals, few have been found to possess activity against *S. aureus* (Kubo *et al*., 1992). Totarol in particular is important as it exhibits activity against both penicillin-sensitive and -resistant strains (Kubo *et al*., 1992). Although the activity of totarol against this bacterium was lower than that against the other bacteria in the study, the MIC and MBC for *S. aureus* have been found to be as low as 1.56 μg/ml (Kubo *et al*., 1992).

1.4.3 Interaction Between Diterpenes and Other Natural Products

The interaction between diterpenes has been investigated by several researchers. Anacardic acid, a naturally occurring phenolic compound, and totarol have similar activities, as both have low minimum inhibitory concentrations (MICs) for MRSA, 6.25 μg/ml for anacardic acid and 0.78 μg/ml for totarol (Muroi and Kubo, 1996). However, anacardic acid has a faster bactericidal activity and is bactericidal for cells at any growth stage, while totarol is bactericidal only for actively growing cells (Muroi and Kubo, 1994; Muroi and Kubo, 1996). The addition of half-MIC anacardic acid to totarol results in a noticeable enhancement of totarol's activity.

A similar interaction was found by Muroi and Kubo (1996) when the antibacterial effects of the two compounds were investigated both independently and as potentiators of the β-lactam antibiotic methicillin. They found that when anacardic acid was combined with methicillin, the MIC for the diterpene was reduced by 128 to 512-fold against the MRSA. Totarol was found to have a similar effect, reducing the MIC by 6-fold for one of the strains used in the study. The MIC for methicillin-sensitive strains was also found to be reduced by the phytochemicals (Muroi and Kubo, 1996).

The reported antibacterial activity of totarol alone was sufficiently high to warrant further investigation for practical use (Kubo *et al.*, 1992). However, the potentiation effect of β-lactam drugs demonstrated by the diterpenes could make them potentially useful as novel adjuncts to antibiotics or other natural products such as anacardic acid for the treatment of MRSA infections. The benefits of combining two or more compounds may include the slower or less likely development of resistance mechanisms in bacteria, as well as enhancing and broadening the total biological activity (Kubo *et al.*, 1992) and decreasing dose-related toxicity as a result of reduced dosage (Muroi and Kubo, 1996). As mentioned previously, the addition of half-MIC anacardic acid can significantly reduce the MIC and MBC for totarol with MRSA, from 1.56 to 0.2 μg/ml even for strains that are multi-resistant to other antibiotics

such as penicillin, tetracycline, erythromycin and chloramphenicol (Kubo *et al.*, 1992).

Frequently plant-derived compounds are overshadowed by the more active, synthetic antibiotics (Muroi and Kubo, 1996). However, the activity exhibited by totarol, with or without the addition of other agents could make it practical for use in therapy. Both totarol and anacardic acid are easily accessible, with totarol being found in a wide range of plants ranging from totara to rosemary to Teatree (Bendall and Cambie, 1995), while anacardic acid is isolated from cashew nut shell oil, cashews and cashew fruit juice which have long been consumed by humans, reducing the possibility of oral toxicity (Muroi and Kubo, 1996).

1.5 Mode of Action of Diterpenes

The suggestion made previously that diterpenes may be used as novel antibiotic agents requires the testing of the compounds to ensure they are not toxic to humans. However, although there have been many studies undertaken on this subject, they have failed to determine a single mode of action. Possible explanations that have been suggested include the inhibition of synthesis of macromolecules, inhibition of cellular respiration and interaction with PBP 2a in *S. aureus.* The relevant literature on each of these possible modes of action is discussed below.

1.5.1 Inhibition of Synthesis of Macromolecules

It has been suggested that diterpenes such as totarol may act by inhibiting the synthesis of protein or DNA. It has been reported that pisiferic acid may act specifically by inhibiting DNA polymerase-α in HeLa-S_3 cells, although RNA and protein synthesis have also been found to be inhibited to a lesser extent in the same cell line (Kobayashi *et al.*, 1989). As discussed above, the C(10) carboxyl group is vital for the retention of this activity in derivatives. This would indicate a mode of action that could potentially be toxic to human cells and as such would lower the

possibility of such a compound as a chemotherapeutic agent for the treatment of humans. The cellular machinery for protein synthesis is also shared by prokaryotes and eukaryotes, rendering any compounds that inhibit it potentially useless as chemotherapeutic agents too.

It has also been suggested that totarol may inhibit peptidoglycan synthesis by interfering with PBP 2a in MRSA (Chamberland *et al.*, 1995). Such inhibition targets a prokaryote-specific process. Chamberland *et al.* (1995) stated that totarol potentiated the activity of β-lactam antibiotics by abolishing the expression or function of PBP 2a, a cell wall protein that is integral in the resistance mechanism of MRSA. This mode of action would have a prokaryote-specific target, and could potentially be safe for humans.

1.5.2 Inhibition of Cellular Respiration

Totarol has been found to be non-toxic to humans at therapeutically achievable levels, while uncoupling oxidative phosphorylation at low concentrations (Evans *et al.*, 1998). This contrasts with the findings of Haraguchi *et al.* (1996) who reported that the probable mode of action of totarol at therapeutic levels was indeed the uncoupling of oxidative phosphorylation.

Haraguchi *et al.* (1996) found that totarol inhibited respiration in *S. aureus, Pseudomonas aeruginosa* and *M. luteus* at concentrations ranging from 0.01 to 0.12 mM. However, data from Evans *et al.* (1998) showed that totarol only caused inhibition of respiration at concentrations above 0.05 mM, which is significantly above the MIC for the compound of 0.007 mM. It was also reported that the uncoupling action the compound exhibited was weak, requiring concentrations of 10 to 100-fold higher than other uncouplers, and that this uncoupling action was probably due to a non-specific interaction with the lipid bilayer. The uncoupling effect that was seen occurred at concentrations up to 0.05 mM and caused a 2-fold increase in coupled respiration, a reasonably weak uncoupling activity. The

uncoupling effect could be seen at any stage of the respiratory chain which further suggested that the effect was a non-specific one, possibly due to the disruption of the lipid bilayer by the accumulation of large amounts of hydrophobic compounds and therefore not the mode of action.

Evans *et al.* (1998) also carried out cytotoxicity tests on several human cell lines to investigate the effect of totarol. Two human tumour cell lines, HeLa and MG 63 and a fibroblast cell line, CH 2983 were used. It was found that totarol was cytotoxic for cells at 25 μg/ml, with cells rounding up and detaching from the plates within 24 hours. However, there was only 50% inhibition of growth at concentrations between 14 and 21 μg/ml (49-73 μM), which is equivalent to 7-10 times the MIC for the compound, and the cells grew normally at concentrations of 30 μM or less, again more than four times the MIC for the compound with *S. aureus*.

1.6 Potential Applications of Totarol

There is potential for totarol to be used against a variety of bacteria. Two test organisms used in this study to include Gram positive and Gram negative bacteria were *H. pylori* and MRSA. For *H. pylori* the application would be as a novel therapeutic agent that could be used independently of other agents.

It is also anticipated that totarol might be used independently as a chemotherapeutic agent for the treatment of MRSA infections. In an *in vivo* trial to investigate the effect of totarol in mice conducted by Evans *et al.* (1998) it was determined that a subcutaneous dose of totarol was protective against a potentially lethal infection with MSSA. However, it was not possible to replicate the trial due to the poor bioavailability of totarol.

However, perhaps more important is the possibility that totarol or other diterpene derivatives might be able to be used in conjunction with existing β-lactam antibiotics to reduce the concentrations of those antibiotics required to kill the bacteria. This is

desirable as currently bacteria such as MRSA can be resistant to β-lactams to such degrees that either the antibiotic cannot be administered in that concentration to the patient, or if it is possible to achieve such high concentrations, they can be toxic to the patient. However, the discovery that totarol can reduce the concentrations of β-lactams significantly and the possible elucidation of the mechanism by which this is achieved (Chamberland *et al.*, 1995; Muroi and Kubo, 1996), provides hope for the treatment of MRSA infections.

Other possible applications for totarol or its derivatives include a topical antiseptic for the treatment of bovine mastitis; a patent has already been taken out for a skin preparation containing totarol as the active ingredient for the control of acne, with favourable results (Shimitani *et al.*, 1990, cited in Bendall and Cambie, 1995). There is also potential for totarol to be used in hospitals for purposes ranging from disinfectant agents to topical applications. Compounds with similar activity to totarol have been found in Teatree and these are used in various products including a cream for burns, although the efficacy of this cream is now in question (Foagali *et al.*, 1997). Totarol has also been shown to exert hypercholesterolemic activity in rats, predominantly by inhibiting cholesterol absorption from the intestine and also possibly by stimulating cholesterol breakdown and excretion. Totarol was found to cause 27 % inhibition (Enamoto *et al.*, 1977, cited in Bendall and Cambie, 1995). A study recently undertaken has also suggested that totarol may prevent lipid peroxidation, which can lead to conditions such as coronary arteriosclerosis and diabetes mellitus due to cellular damage caused by membranes with decreased fluidity and subsequently disrupted membrane structure and function (Haraguchi *et al.*, 1997; Haraguchi *et al.*, 1996).

1.7 *Helicobacter pylori*

1.7.1 *Helicobacter pylori*

Discovered in 1983 by Dr Barry Marshall and subsequently cultured and named *Helicobacter* rather than *Campylobacter* (Stewart Goodwin *et al*., 1989), *Helicobacter pylori* (*H. pylori*), was one of the Gram negative test organisms included in this work. It is a Gram negative, curved bacterium determined to be the causative agent of gastric ulcers and has been implicated in gastric cancer. Its discovery has made it possible to explain the occurrence of symptoms including gastritis, stomach ulcers and gastric cancer, indicating environmental conditions and diet may not be their only causes (Dunn *et al*., 1997, NIH Consensus Conference, 1994).

H. pylori can be found in any part of the stomach, but most commonly in the antrum. The appearance of the bacteria is almost always linked to inflammation near the surface epithelium (Dunn *et al*., 1997). In early stages of gastritis there is a marked infiltration of the mucosa by neutrophils and exudate appears on its surface (Dixon, 1995; Wyatt and Gray, 1992). As the infection proceeds, mucous is lost and the cells become uneven (Goodwin, 1991). A chronic infection is characterised by an increase in lymphocytes and plasma cells in the lamina propria. Gastritis is typically confined to the antrum, although it can involve the whole stomach, in which case it is called pangastritis. This is typically seen in developing countries.

H. pylori has world-wide distribution and a number of countries with high rates of gastric cancer are now known to also have high levels of *H. pylori* infection (Gasbarrini *et al*, 1995). As a result, it may be possible to prevent gastric cancer by eradicating *H. pylori* from the stomach (McFarlane and Munro, 1997).

1.7.2 Treatment Problems Associated with *H. pylori*

There are problems associated with *H. pylori* treatment regimes. The majority of treatments for the bacteria are triple, usually consisting of two antibiotics such as tetracycline and metronidazole, and bismuth salts (Logan *et al.*, 1993). Bismuth salts have been an essential part of the treatment but are not approved for human therapy in all parts of the world, their omission lowering the efficacy of the regime (Logan *et al.*, 1993). An additional problem occurs with the emergence of metronidazole-resistant strains of *H. pylori* (Logan *et al.*, 1993). Rates of metronidazole resistance in strains of *H. pylori* range from 6.4 to 84 % throughout the world, indicating it is a widespread problem (Xia *et al.*, 1994). These factors when coupled with the fact the regimes require a long treatment period and the frequent occurrence of side effects which can range from alterations in taste to vomiting and diarrhoea (Logan *et al.*, 1993) reduce the success rate of treatment as often patient compliance is lowered and regimes are not completed. In developing countries in particular, where the benefits of such programmes would be the greatest, there are much lower success rates due to wide-spread resistance to metronidazole, and also the economic constraints of the countries (McFarlane and Munro, 1997). Consequently there is a real need to find alternative therapies for *H. pylori*.

Several new drugs have been tested for their activity against *H. pylori* and they are of two main types; novel antibiotics such as clarithromycin (Logan *et al.*, 1993) and other compounds such as proton pump inhibitors, including omeprazole (Logan *et al.*, 1993) and lansoprazole (Parente et al, 1995). However, in most cases there is still the need for dual, triple or even quadruple therapies (Logan *et al.*, 1993; Parente et al., 1995), although the length of time patients must be treated has been lowered. A combined therapy of omeprazole in combination with metronidazole and either clarithromycin or amoxycillin has been found to eradicate *H. pylori* in 90 % of cases, with only one week of treatment (Labenz *et al.*, 1995). The advent of proton pump inhibitors has provided a new tactic in the treatment of gastric ulcers caused by *H. pylori*. Their mode of action is to raise the pH of the stomach by suppressing the acid secretion of parietal cells. Studies on the activity of omeprazole have led to the

suggestion that it may also decrease the rate of breakdown of antimicrobial agents in the stomach (Logan *et al.*, 1993).

1.8 Methicillin-resistant *Staphylococcus aureus* (MRSA)

1.8.1 The Origins of MRSA

Staphylococcus aureus (*S. aureus*) has long been recognised as a common human pathogen (Kluytmans *et al,* 1997). It is the causative agent of many minor infections in healthy individuals such as boils or food poisoning, but infections in hospital patients can have severe consequences despite antibiotic therapy (Kluytmans *et al.,* 1995; Yzerman *et al.,* 1995).

The introduction of the semi-synthetic β-lactam antibiotic methicillin in 1959 and 1960 (reviewed by Chambers, 1988) provided a means of controlling the pathogen briefly. However, almost immediately resistant strains were observed (Schmitz and Jones, 1997) although these were generally thought to be confined to laboratory strains and not of any real clinical significance (Chambers, 1988). Resistance was noted in coagulase-negative strains before *S. aureus* (Stewart, 1961) although it quickly spread to this species. Initially resistance was heterogenous, with only one cell in 10^8 expressing resistance. However, the proportion of resistant cells could be dramatically increased by just one passage in the presence of methicillin (Chambers, 1988).

The first nosocomial outbreak of methicillin-resistant *S. aureus* (MRSA) occurred in 1963 (Chambers, 1988). This strain was unlike previously isolated ones because it displayed more uniform or homogenous growth in the presence of methicillin, and was also resistant to cephalosporins, oxacillin and cloxacillin (Chambers, 1988). The strain was much more similar to strains of MRSA seen today that are resistant to almost all common antibiotics (Maple, *et al.*, 1989).

There have been numerous outbreaks of MRSA reported in Europe from the 1960's (Jesson *et al.,* 1969, Benner and Kayser, 1968) and the USA from the 1970's (Crossley *et al.,* 1979, Crossley *et al.,* 1979) and strains have become established outside hospitals, particularly among intravenous drug users. MRSA have now become endemic in many hospitals throughout the USA, causing life-threatening infections in hospitalised patients (Irizarry *et al.,* 1996). Today multiply resistant strains of MRSA account for 11-40 % of all *S. aureus* strains; in the USA the proportion of MRSA strains has rapidly increased from less than 5 % in the early 1980's to 29 % in 1991 (Panlilio *et al.*, 1992). Generally the multiply resistant strains are nosocomial, thus presenting a serious therapeutic problem (Brumfitt and Hamilton-Miller, 1989) for immunocompromised patients. It is therefore very important to be able to effectively treat infections in such patients. However, many strains of MRSA are now resistant to almost all commonly used antibiotics, including β-lactams, macrolides and aminoglycosides (Maple *et al.,* 1989; Brumfitt and Hamilton-Miller, 1989). As a result infections are becoming increasingly difficult to treat.

1.8.2 Treatment Problems Associated with MRSA

Due to the increasing number of infections caused by MRSA, many strains of which are now multi-resistant (Ayliffe, 1997), therapy has become problematic (Voss and Doebbling, 1995). Strains of MRSA resistant to ciprofloxacin are becoming increasingly prevalent; only 16.7 % of MRSA strains were found to be sensitive to the antibiotic in a 1992 survey, although resistance was extremely low only a few years previously. The rate of ciprofloxacin resistance is now expected to mirror the rate of methicillin resistance (Baron, 1995). Currently the glycopeptide antibiotic vancomycin is the only reliable agent to treat infections caused by MRSA strains (Liu *et al.*, 1996; Palmer and Rybak, 1996; Blumberg *et al.,* 1991). There are no naturally occurring strains of MRSA that are resistant to the bacteriostatic effects of vancomycin, although some strains are resistant to its bactericidal activity (Mlynarczyk, *et al.,* 1997). Until very recently all strains of MRSA were sensitive to vancomycin although resistance had been observed in Enterococci and coagulase-

negative Staphylococci and resistance has been seen to be transferred from Enterococci to *S. aureus* (Noble *et al.,* 1992). The discovery of an antimicrobial with a mode of action differing from that of vancomycin is therefore very desirable (Kato *et al.,* 1997).

The use of vancomycin has dramatically increased world-wide recently, with the increasing rates of MRSA, and at the same time resistance in Gram positive organisms has also dramatically increased (Kluytmans *et al.,* 1997). This is clearly seen in intensive care units in hospitals in the USA where in 1989 less than 5 % of Enterococci were resistant, but in 1992 almost 8 % were resistant (Grace Emori and Gaynes, 1993). The possible transfer of resistance mechanisms from Enterococci to *S. aureus* is of great importance as it would result in a totally resistant organism, dramatically affecting hospitalised patients (Kluytmans *et al.,* 1997). Transfer of these resistance mechanisms had been shown *in vitro* and in animal studies, and in 1996 the first case of a vancomycin-resistant strain was seen in Japan (Hiramatsu *et al*., 1997).

Although vancomycin still remains the antibiotic of choice for MRSA infections, there are major concerns about the increasing use of this drug; vancomycin has a very narrow toxic to therapeutic ratio, with toxic side effects resulting from high doses; it must be administered intravenously, resulting in hospital admission, increasing the related cost of treatment; and as stated previously, the growing concern about the development of vancomycin resistant strains (Schaad *et al.,* 1994). Other problems also exist with vancomycin, including the fact that it is less rapidly bactericidal than other antibiotics such as nafcillin (Small and Chambers, 1990).

Because of the above problems with vancomycin, the drug is often administered in conjunction with another antibiotic such as rifampin or gentamycin (Mulazimoglu *et al.,* 1996), although this is also problematic as the combination is more nephrotoxic than vancomycin alone (Fauconneau *et al.,* 1992). Alternative treatments for MRSA infections involve the addition of β-lactamase inhibitors with penicillinase-susceptible agents to help prevent the hydrolysis of the antibiotics (Fasola *et al.,*

1995). Other antibiotics such as fluroquinolones, muprocin, or combinations such as trimethoprim-sulfamethoxazole are also used in some cases, although none of these treatment strategies are more effective than vancomycin (reviewed by Chambers, 1997). It is also difficult to rid hospitals of MRSA as many strains are more resistant to both chronic and acute exposures to antiseptics and disinfectants (Irizarry *et al.*, 1996).

Most MRSA strains possess two main mechanisms of resistance; the production of β-lactamase which hydrolyses the drugs, and PBP 2a, an altered penicillin binding protein with very low affinity for β-lactam antibiotics which still catalyses cell wall synthesis in the presence of antibiotics (Chambers and Hackbarth, 1987). However, a third mechanism of resistance has also been discovered; some strains have modifications to their normal PBPs resulting in lower affinity for β-lactam antibiotics (de Lencastre *et al.,* 1994; Chambers, 1988).

In 1996, the description of a vancomycin resistant strain of MRSA was first reported with the isolation of a strain with an MIC for vancomycin of 8 mg/L (8μg/ml) (Hiramatsu *et al,* 1997). However the mechanism of resistance in this strain was not determined to be by the mechanisms possessed by Enterococci; rather it was suggested it may be due to an intrinsic mechanism of augmented cell wall synthesis, indicated by factors such as a cell wall that was twice as thick as normal, and increased production of both PBP 2 and PBP 2a (Hiramatsu *et al.,* 1997). This indicates that transfer of resistance from Enterococci may not be the only way for vancomycin resistance to develop; the bacteria may develop resistance independently.

1.9 Local Background Leading to This Research

The initiation of this research project occurred when Dr Gary Evans, a synthetic chemist at Industrial Research Limited (IRL), Lower Hutt, approached researchers for bacteriological support in the testing of his derivatives of totarol and other diterpenes.

Derivatives are synthesised by Dr Evans at IRL and tested for efficacy and spectrum in this study. The majority of derivatives to be tested in this research were synthesised from totarol, although five diterpenes from species of pine, and eight compounds derived from podocarpic acid, a diterpene similar in structure to totarol, were also included. Derivatives of totarol and podocarpic acid varied in the structural features of their aromatic ring and/or carboxyl group. It was hoped that this research would identify derivatives that are effective against both Gram positive and Gram negative bacteria, with increased antibacterial properties. Findings from this work could potentially be used to aid in the elucidation of the structure-function relationship for this group of compounds, enabling the future synthesis of derivatives with increased activity and bioavailability. An understanding of the mode of action of these compounds will also aid in future development of bioactive compounds for the treatment of the bacteria discussed above.

1.10 Objectives of This Research Project

1. To identify derivatives of totarol that are effective against *H. pylori* and MRSA independently
2. To identify derivatives that potentiate the activity of β-lactams with MRSA
3. To investigate the mode of action of the most effective diterpene derivative. Possible modes of action to be included in this study:

    -Inhibition of peptidoglycan synthesis

    -Inhibition of DNA synthesis

    -Inhibition of protein synthesis

    -Interaction with PBP 2a

    -Inhibition of respiration or uncoupling of oxidative phosphorylation
4. To determine the cellular target of the most effective derivative

2. MATERIALS AND METHODS

2.1 Bacterial Strains

2.1.1 Sources

Four bacterial strains were used in this research. *H. pylori* strain CCUG 17874 (Culture Collection of the University of Götenburg, identical to NCTC 11637, the type strain), was obtained from stocks held in the lab. Two strains of *S. aureus* were used; strain 87 (Oxford strain), used as a methicillin-sensitive representative strain and strain MR96/164, which was resistant to β-lactam antibiotics, were both obtained from the culture collection held at the Communicable Disease Centre, Porirua, New Zealand. *E. coli* strain C600 (genotype F^-[e14$^-$(McrA$^-$) or e14$^+$(McrA$^+$)] thr$^-$ *leuB6 lacY1 supE44 rfbD1 fhuA21*. Bachmann, 1987, pp 1190-1219, *in E. coli* and *Salmonella typhimurium*, Cellular and Molecular Biology, F. C. Neidhardt (Ed.), ASM, New York; Raleigh *et al.*, 1989, *in* Current Protocols in Molecular Biology. F. M. Ausubel *et al.* (Eds.). Publishing Associates and Wiley International, New York) was obtained from stocks held within the lab.

2.1.2 Maintenance of Bacterial Strains

H. pylori was maintained on Columbia Base agar plates containing 5 % defibrinated horse blood. Cultures were subbed on to fresh plates every 2 to 3 days to maintain active growth. *S. aureus* and *E. coli* strains were maintained on tryptic soy agar plates. All bacterial strains were kept at -80°C in 40 % glycerol as stocks.

2.2 Bacteriological Media

2.2.1 Columbia Base Agar Containing 5 % Defibrinated Horse Blood

This media contained (g/400 ml): Columbia base agar (CBA), 15.6; and 20 ml defibrinated horse blood, in 380 ml Milli-Q water. CBA and water were autoclaved at 121°C for 15 minutes. The agar was then cooled to 70°C before the blood was added aseptically. The agar was stirred for around 15 minutes until the agar became a chocolate colour, before pouring into plates. Agar was surface dried to reduce fungal contamination before the plates were stored at 4°C.

2.2.2 Tryptic Soy Agar Plates (TSA)

This media contained (g/L): Tryptic soy broth, 30; agar, 15. The media was sterilised by autoclaving at 121°C for 15 minutes. After cooling, the agar was poured into plates and stored at 4°C until use.

2.2.3 Luria Broth (LB)

A modified Luria broth was made as described by Chamberland *et al* (1995). This contained (g/L): Tryptone, 10; and yeast extract, 5. 10 g/L NaCl was omitted from the usual recipe. The broth was sterilised by autoclaving at 121°C for 15 minutes. Broth in use was stored at 37°C in order to rapidly detect contamination.

2.3 Preparation and Storage of Diterpenes

Diterpenes were received from Dr Gary Evans, at Industrial Research Limited, in powder form and dissolved in methanol, to give concentrations ranging from 15 to 70 mg/ml, depending on the amount received. Diterpenes were aliquoted into 1 ml volumes and stored at -20°C until use, as were stock solutions.

2.4 Preparation and Storage of Antibiotic Solutions

100 mg/ml stock solutions of methicillin, imipenem, penicillin G and ampicillin were prepared by dissolving the appropriate amount of antibiotic in Milli-Q water. Solutions were sterilised by passage through a sterile 0.45 μm filter. Solutions were stored at -20°C.

2.5 MIC Determination

The minimum inhibitory concentration (MIC) for each of the diterpenes with both strains of *S. aureus* were determined by the standard macrobroth dilution method, slightly modified from that described in the Manual of Clinical Microbiology (6th Ed),(P. R. Murray (Ed.), ASM Press, Washington D.C.) An overnight culture of *S. aureus* was diluted 100-fold and grown until the OD_{600} reached 0.6, as measured on a spectrophotometer. Serial two-fold dilutions of the diterpene were prepared in LB to give final volumes of 2 ml. These and a positive control of 2 ml LB were inoculated with 50 μl of culture and then incubated shaking at 37°C for 24 hours before the MIC was read. The MIC was determined as being the lowest concentration of diterpene that resulted in complete inhibition of growth, as judged by eye. Thus the lowest concentration resulting in no turbidity was determined to be the MIC for the diterpene.

This method was also carried out for each of the antibiotics with *S. aureus* in order to determine their MIC which could then be referred to in the potentiation experiments. *E. coli* was also used in this method to determine the MIC for the derivatives with a Gram negative organism.

2.6 MBC Determination

The minimum bactericidal concentration (MBC) for each diterpene was determined by spreading a loopful of all the concentrations that resulted in no apparent growth in the MIC determination on TSA plates and incubating at 37°C. The MBC was determined as the lowest concentration of diterpene that yielded no bacterial growth after 24 hours incubation.

2.7 Disk Diffusion Technique

Due to the difficulty of growing *H. pylori* in liquid culture, the disk diffusion technique was employed to determine whether any of the diterpenes showed significant activity against a Gram negative bacterium. The method used was modified from that outlined in the Manual of Clinical Microbiology (6th Ed.) (P. R. Murray (Ed.), ASM Press, Washington D.C.) and is described below.

2.7.1 Preparation of Antibiotic Disks

Antibiotic disks were sterilised by baking at 160°C for 2 hours. They were stored in an airtight container until their use. Prior to their application to the agar plates in the test they were inoculated with 20 µl of diterpenes that had been diluted in methanol to give final concentrations of 250, 125, 25, 5 and 0 µg/ml on the disks. This resulted actual amounts of diterpene on the disks of 50, 25, 5, 1 and 0 µg. Disks were left to dry in the sterile conditions of the laminar flow cabinet before being applied to the agar.

2.7.2 Preparation of *H. pylori*

A 2-3 day old plate culture of *H. pylori* showing good growth was swabbed into 5 ml TSB that had been pre-warmed in an incubator with 5 % CO_2, so as to be optimal for the survival of *H. pylori*. The optical density was determined and if necessary

adjusted to give on OD_{600} of 0.6. 0.5 ml of this culture was then used to flood an agar plate, and the excess drained off. Plates were dried in the laminar flow cabinet for 5 minutes before the pre-dried disks containing diterpenes were added to the plates. Disks were firmly pressed onto the agar, as evenly spaced as possible, with a pair of sterile forceps to ensure they did not move during incubation. Plates were then incubated at 37°C with 5% CO_2 for 48 hours. After 48 hours the diameter of the zone of inhibition around each disk was measured including the disk itself.

2.8 Potentiation of β-lactam Antibiotics

The method by which the extent to which each of the diterpenes reduced the MIC of the β-lactam antibiotics tested was determined was essentially the same as that used for MIC determination. Serial 2-fold dilutions of the antibiotics were prepared in a final volume of 2 ml, ranging from the MIC downwards. A positive control containing no antibiotic and a negative control containing no bacteria were also included. The diterpene was added to each of the test concentrations to give a final concentration of 10 μg/ml if this was less than the MIC, or at half the MIC if this was smaller. 50 μl of *S. aureus* prepared as in Section 2.5 was added to each tube, and all tubes were then incubated shaking at 37°C for 24 hours, after which time the MIC was determined as in Section 2.5. The MBC was also determined for each compound as described in Section 2.6.

MIC determination for methicillin in the presence of diterpene was also conducted using *E. coli* in order to determine whether the derivatives possessed any potentiation activity against a Gram negative bacterium. *E. coli* was used in place of *H. pylori* due to the difficulties of growing it in liquid culture.

2.9 Incorporation Assays

The effect of totarol on the synthesis of three macromolecules, DNA, protein and peptidoglycan, was investigated using the incorporation of radioactively labelled precursors.

2.9.1 Materials

2.9.1.1 Trichloroacetic Acid

A 10 % solution of trichloroacetic acid (TCA) was made by dissolving 40 g of the acid in 400 ml of Milli-Q water, while a 5 % solution was made by dissolving 20 g of the powder in the same volume of Milli-Q. Both solutions were stored at 4°C until used.

2.9.1.2 Sodium Dodecyl Sulphate

A 10 % solution of sodium dodecyl sulphate was made by dissolving 10 g of the detergent in 100 ml of Milli-Q water

2.9.1.3 Isotonic Saline

The solution contained (g/L): NaCl, 9 dissolved in 1 L of Milli-Q water. The solution was sterilised by autoclaving at 121°C for 15 minutes.

2.9.1.4 0.1M Hydrochloric Acid (HCl)

A 0.1 M solution of hydrochloric acid was made by diluting 1.6 ml of concentrated hydrochloric acid in a final volume of 200 ml Milli-Q water.

2.9.1.5 Scintillation Fluid

Scintillation fluid contained (g/L): diphenyl oxazole (PPO), 4, dissolved in 667 ml toluene and 333 ml Triton-X.

2.9.2 DNA Incorporation Assay

The measurement of the effect of totarol on DNA synthesis is modified from that of Powell *et al.* (1992).

2.9.2.1 Preliminary Experiments

A preliminary experiment was conducted before the use of any isotope in order to determine the effective MIC for the conditions of the experiment. These differed slightly from those of the macrobroth dilution as cultures were incubated in a stationary waterbath and were only shaken at sample times. Cultures were grown in identical conditions to those used in the actual experiment and totarol was added to them to give a range of final concentrations up to that of the MIC determined in the macrobroth dilution experiment. The MIC was determined for these conditions in the same manner as previously described in Section 2.5 after the overnight incubation of the cultures. Under these conditions the effective MIC was found to be 1.5 μg/ml. However, a concentration that inhibited the growth of the bacteria, not killing them completely was desirable so as to be able to measure the rate of isotope incorporation. 1.25 μg/ml was selected as the highest concentration used in subsequent incorporation experiments to ensure that there would still be growth and therefore incorporation if the cellular machinery was intact.

A range of experiments were also conducted to determine the dilution factor of the overnight culture that resulted in the most vigorous cell growth over the three hours of the experiment, allowing the cultures to reach exponential growth phase with as many cellular divisions as possible.

5 ml cultures diluted at the different factors were incubated over a three hour period under the same conditions of the experiment. Samples were taken at thirty minute intervals, diluted in LB and plated on TSA before being incubated overnight at 37°C. The concentration of bacteria in each culture was calculated and a growth curve showing CFU/ml versus time plotted. A 200-fold dilution of the overnight was selected as the optimal dilution factor.

2.9.2.2 Incorporation of Radiolabelled Precursor

An overnight culture of MRSA strain MR96/164 was diluted 200-fold to give six 5 ml cultures. Tritiated methyl-thymidine(Amersham, 1μCi/ml, specific activity 37 Mbq/ml) was added to a final concentration of 2 μCi/ml and mixed thoroughly through the culture. A 0.5 ml sample was immediately removed to an Eppendorf tube and frozen in liquid nitrogen, and the time registered as 0 min. The remainder of the cultures were incubated in a waterbath at 37°C until the next sample timepoint.

Samples were removed at 30 minute intervals for 180 minutes, and an additional sample at 105 minutes was also added in later experiments for more accurate determination of the effect of totarol on the incorporation of labelled precursor. At 90 minutes totarol was added to 5 of the tubes to give final concentrations of 0.25, 0.5, 0.75, 1.0, and 1.25 μg/ml. The sixth tube was a positive control to which totarol was added. All tubes were thoroughly mixed using a vortex mixer at each time point to keep cultures well-aerated.

Samples were immediately frozen in liquid nitrogen to prevent further growth and stored at -20°C until processed.

2.9.2.3 Precipitation of Labelled DNA

Samples were thawed at room temperature and 0.1 ml of 10 % SDS was added to each before being boiled for 2 minutes. Each sample was briefly spun in a benchtop centrifuge to bring the contents down from the lid so as to prevent any splashing of radioactive contents when the tube was opened, and 0.9 to 1.0 ml of ice-cold TCA was added to all tubes. The samples were then kept on ice for 60 minutes to precipitate the DNA in each.

After precipitation each sample was filtered using vacuum filtration onto Whatman GF/C glass filters, rinsing each one through with 1 to 2 ml ice-cold 10 % TCA. Filters were placed in Wheatman glass scintillation vials and baked for at least 60 minutes with their lids loosened until thoroughly dry.

2.9.2.4 Measurement of Incorporation

The rate of incorporation of ^{3}H-methyl thymidine into the DNA of samples was determined by adding 3 ml of scintillation fluid to each vial and carrying out 1 minute counts on each in a Beckman scintillation counter. Incorporation was expressed as counts per minute per ml of culture.

2.9.2.5 Measurement of Culture Growth During Incorporation Experiment

Three methods were used to obtain a measure of the growth of the cultures during the incorporation:

1. Viable cell count

Before undertaking the incorporation assays, growth curves were completed to calculate the cell densities of the cultures at the various concentrations of totarol and over the three hour period of the experiment. Dilutions to provide 30 to 300 colonies per plate were calculated and used in the viable cell counts.

At each time point during the incorporation experiment 0.1 ml of culture was removed in addition to the 0.5 ml for scintillation counts. This was diluted from 10^4 to 10^5 depending on the concentration of totarol and the time in the experiment, in modified LB (Section 2.2.3), and 10 μl of each sample plated on TSA plates (Section 2.2.2). Plates were incubated overnight at 37°C and the resulting colonies counted the following day. The concentration of viable cells/ml was calculated for each tube at each sample time and incorporation of ^{3}H-methyl thymidine calculated and expressed as counts/min/viable cell.

2 Determination of dry weight

Initially 0.1 ml of culture was sampled in addition to the 0.5 ml for scintillation counts. This was later increased to 0.5 ml and 0.1 ml was sampled for scintillation counts as the first volume did not yield sufficient dry weight to be measured accurately. It was necessary to reduce the volume sampled for scintillation counts as there was a limit on the amount of isotope that could be purchased, so it was necessary to limit the volume of cultures to 5 ml.

Each sample was immediately filtered by vacuum filtration onto 0.45 μm nitrocellulose filters that had been pre-weighed in Eppendorf tubes and was washed through with 1 to 2 ml sterile isotonic saline. All filters were dried overnight in Eppendorf tubes using the freeze-drying apparatus in the Department of Biochemistry, Massey University, to remove all liquid from the samples.

After drying, the filters and Eppendorfs were re-weighed and the dry weight of samples calculated.

3. Measurement of total protein

Two assays were utilised in the determination of total protein. Initially 0.2 ml of culture was removed at each time-point and 0.3 ml was removed for scintillation counts. In later experiments 1 ml of sample from a parallel, non-labelled culture was removed at each time-point.

Parallel cultures

The use of parallel cultures became necessary for the same reason as discussed above; there were financial limitations on the amount of isotope that could be purchased, limiting the volume of labelled cultures to 5 ml. 5 ml cultures were prepared at the same time as that receiving the isotope, from the same overnight culture. All cultures were grown up together over the same three hour period in the same waterbath. Samples were removed at the same time as the labelled culture and totarol was added at 90 minutes to give the same final concentrations as for the labelled cultures.

All samples removed for protein determination were immediately frozen in liquid nitrogen to prevent further growth and stored at -20°C until the assay was carried out.

The determination of total protein was carried out using the Bio-Rad Protein assay kit, based on the method of Bradford (1976). Bovine serum albumin (BSA) was used as a positive control and standards of known concentration were made by diluting BSA (1.45 mg/ml) in LB which had been boiled for 10 minutes. The concentrations of standard used depended on the particular assay being used; for the microassay for microtitre plates they ranged from 8 to 80 μg/ml, while the standard microassay procedure produced a standard curve linear for 1 to 10 μg protein/ml, so standards were prepared to cover this range.

All samples were boiled for 10 minutes to lyse the cells before the addition of the dye reagent. For the microassay procedure using microtitre plates, 40 μl of undiluted dye reagent was added to 160 μl of sample, while in the standard microassay 200 μl of undiluted dye reagent was added to 800 μl of sample, thoroughly mixing the dye into the samples after its addition. The same dye to sample ratios were also used for standards.

After the addition of dye all samples and standards were incubated for the same period of time, being between 5 and 60 minutes as according to manufacturers instructions. The absorbances of all tubes were then read at 595 nm on a bench spectrophotometer. Absorbances were read in a staggered fashion so as to ensure all tubes had been incubated for the same period of time. The absorbances of standards were used to plot a standard curve showing absorbance at 595 nm versus protein, in μg/ml. The standard curve was then used to determine the concentration of protein in each sample, corresponding the absorbance of each to the concentration of protein resulting in that absorbance. The total protein in each sample was then expressed as μg/ml and incorporation of thymidine under the varying concentrations of totarol was calculated and expressed as scintillation counts/min/μg protein.

2.9.3 Protein Incorporation Assay

The method used for this assay was modified from that of Mastersi and Hong (1981), but was essentially the same as that employed for DNA incorporation. Tritium-labelled glutamine (Amersham, 1 μCi/ml, specific activity 37 MBq/ml) was the precursor used to measure protein synthesis over the three hour period. Cultures were prepared and samples taken in the same way as discussed above (Section 2.9.2.1). The precipitation of labelled protein differed in methodology from that of labelled DNA. The 2 minute boiling in 10 % SDS was omitted and 1.0 ml 10 % ice-cold TCA was added to thawed samples which were then kept on ice for 60 minutes to precipitate the protein. Filtration was by the same methodology as for DNA, (Section 2.9.2.2), although 5 % TCA was used to rinse samples through the filters and was followed with an additional rinsing of about 5 ml of 0.1 M HCl for each sample (Mastersi and Hong, 1981). Incorporation per viable cell and per microgram of protein was calculated (Section 2.9.2.4).

2.9.4 Peptidoglycan Incorporation Assay

Incorporation of tritiated N-acetyl glucosamine (NAG) (Amersham, specific activity 1.65 GBq/mg) was used to measure the synthesis of peptidoglycan in the presence of increasing concentrations of totarol. The methodology of labelling the cultures, sampling and storage of the samples was the same as described for the previous two assays (Section 2.9.2.2) However, as there was no previous method to use for the recovery of the labelled peptidoglycan, a pre-experiment was conducted to determine the most efficient method to collect the labelled peptidoglycan on the glass filters for scintillation counts.

Triplicate cultures were prepared by diluting an overnight culture 200-fold. ^{3}H-NAG was added to each culture to a final concentration of 2 μCi/ml, and the cultures incubated in the normal manner in a stationary waterbath at 37°C. 0.5 ml samples were removed at 45 minute intervals over 180 minutes and immediately frozen in liquid nitrogen before processing.

Three methods were used to isolate the labelled peptidoglycan on glass filters:

1. Each sample from the first culture was simply filtered onto glass filters by vacuum filtration and washed through with 1 to 2 ml sterile isotonic saline.
2. Each sample from the second culture was boiled in 10 % SDS for 2 minutes to lyse the cells prior to being filtered through glass filters, washing with 1-2 ml sterile isotonic saline.
3. Each sample from the third culture was boiled in 10% SDS for 2 minutes before being spun for 15 minutes at 14000 rpm. The supernatant in each tube was poured off and the pellet resuspended in 1 ml sterile isotonic saline before being filtered, again washed through with 1 to 2 ml saline.

The method providing the highest recovery rate of labelled peptidoglycan, the second described above (see results Section 3.7.1), was used in future incorporation assays.

A control experiment was conducted to ensure synthesis of peptidoglycan was being measured, using a negative control to demonstrate inhibition of peptidoglycan synthesis. The MIC for vancomycin with MRSA strain MR96/164 was determined by the method described in Section 2.5. Vancomycin was added to give a final concentration of 1 μg/ml (half-MIC) at the beginning of the experiment and samples were taken every 45 minutes. Each sample was boiled for two minutes before filtration.

Subsequent incorporation experiments were conducted with modifications to the method (Sections 2.9.2.1 to 2.9.2.3) being in precipitation of labelled peptidoglycan. Incorporation was expressed as scintillation counts/min/μg protein.

2.10 Investigation of the Effect of Totarol on Cellular Respiration

2.10.1 Materials

2.10.1.1 Glucose/Salt Solution

This solution was made according to the recipe given by Dr Jan Schmid (personal communication) and contained (g/l): Glucose, 30; and NaCl, 9. The solution was made by dissolving the two solids in Milli-Q water and was sterilised by autoclaving for 15 minutes at 121°C. This solution has a concentration of 7.9 μl oxygen/ml when saturated with atmospheric oxygen.

2.10.1.2 Cyanide

A 1 M solution of potassium hexacyanoferrate was prepared by dissolving 422.4 mg in 1 ml sterile Milli-Q water. The solution was stored at room temperature and was freshly prepared every 2 days.

2.10.2 Preparation of Bacteria

An overnight culture of MRSA was diluted 50-fold in Luria Broth (Section 2.2.3) to give a final volume of 5 ml. The culture was grown shaking at 37°C until the OD_{600} reached 0.6.

2.10.3 Preparation of Oxygen Electrode

3 ml of the sterile solution described in Section 2.10.1.2 was placed in the chamber of the oxygen electrode which had been pre-heated to 37°C by a pumping waterbath and the solution vigorously mixed for 20 minutes using the magnetic stirrer in the chamber to saturate the solution with oxygen. During this time the probe was fitted with a new membrane, ensuring saturated potassium chloride had been poured over the electrode before the membrane was attached. The functioning of the probe was tested daily to ensure accurate measurement of oxygen levels. After 20 minutes of aeration the stirrer was turned off and the probe carefully placed in the chamber so as

to exclude any air bubbles which can affect oxygen readings, and the magnetic stirrer restarted. When the reading on the meter had stabilised giving a steady reading of oxygen saturation, the meter was calibrated to read 100 %. The 0 % reading was also calibrated by setting the meter to the zero position and ensuring the meter read 0 % oxygen content. The saturated solution was then removed from the chamber.

2.10.4 Measurement of Cellular Respiration

4.5 ml of the above solution (Section 2.10.1.1) was placed in the chamber with 0.5 ml of cells (Section 2.10.2). The suspension was stirred for 3 minutes with the probe removed in order to equilibrate the temperature of the solution with that of the chamber. After 3 minutes, the probe was carefully placed in the chamber, forcing all air bubbles out of the solution, and respiration was observed. When the oxygen consumption by the bacteria had reached a steady rate, measurement of respiration began. Steady-state respiration was measured for a period of 5 or 10 minutes before totarol was added.

2.10.5 Addition of Totarol

When 5 or 10 minutes of steady-state respiration had been achieved, totarol was added to the chamber using a length of tubing attached to a pipette tip and a 20 μl pipette. This was inserted into the chamber by sliding it down the groove present on the side of the electrode, into the chamber. This allowed the addition of totarol without having to stop the spinning of the solution and removing the probe, thus introducing more oxygen into the solution when reinserting the probe. The volume of totarol added was always 17.5 μl to minimise any effects due to the volume added in each experiment. Totarol was added to give final concentrations of 3.5×10^{-2}, 7×10^{-3}, 3.5×10^{-3}, 1×10^{-3}, 7×10^{-4}, 3.5×10^{-4}, 7×10^{-5} and 0 mM. It was originally decided to also include 0.1 mM, but this did not prove to be possible. The concentrations tested covered the range tested in the literature discussed in Section 1.8, where researchers found conflicting effects on respiration due to totarol. Each concentration of totarol was tested in triplicate.

After the addition of totarol a further 10 minutes of steady state respiration was measured.

2.10.6 Addition of Cyanide

After steady-state respiration in the presence of totarol had been observed, cyanide was added at a concentration previously determined to be inhibitory to respiration to indicate that the bacteria had indeed been respiring.

100 μl of the potassium hexacyanoferrate solution (Section 2.10.1.2) was added to the chamber by the same method as for totarol, using a fresh Section of tubing and a 200 μl pipette. The rate of respiration was again measured for a 10 minute period after the addition of the cyanide solution.

After each experiment the chamber and probe were thoroughly rinsed to remove any traces of cyanide that may inhibit the growth of the cells in subsequent experiments. At the conclusion of each experiment the rate of oxygen consumption was calculated using the known concentration of oxygen in the NaCl/glucose solution, and expressed as microlitres of oxygen consumed per minute per microgram of dry weight of bacteria. The determination of the dry weight is described in the following Section.

2.10.7 Determination of Dry Weight of Culture

An overnight culture of MRSA was diluted 50-fold as for respiration experiments to a final volume of 500 ml and grown shaking at 37°C. 11 ml samples were removed every 15 minutes for three hours. At each sample time 1 ml was immediately removed to determine the optical density of the culture. The remaining 10 ml was filtered in duplicate 5 ml volumes under vacuum filtration onto nitro-cellulose filters with a porosity of 0.45 μm that had been pre-weighed. Samples were rinsed through with 1 to 2 ml sterile saline (Section 2.9.1.3) before being freeze-dried overnight.

Filters were re-weighed and the dry weight per ml calculated for each sample. A graph showing dry weight versus optical density was plotted and used in subsequent experiments to determine the dry weight per ml of each culture, and thereby express oxygen consumption as microlitres of oxygen consumed per minute per microgram of dry weight.

2.11 Investigation of the Effects of Totarol on PBP 2a

This protocol, providing a non-radioactive labelling of PBP 2a was conducted as according to Dargis and Malouin (1994) although the final step of the detection procedure differed slightly, using the ECL DNA detection kit rather than the ECL Western Blotting kit.

2.11.1 Materials

2.11.1.1 Phosphate Buffered Saline (PBS) (pH 7.0)

A 50 ml solution of PBS was made by combining 30.5 ml of 0.2 M Na_2HPO_4 and 19.5 ml 0.2 M NaH_2PO_4. The solution was autoclaved at 121°C for 15 minutes to sterilise it and stored at room temperature until use.

2.11.1.2 0.1M Sodium Phosphate Buffer (pH 7.0)

1 L of sodium phosphate buffer was prepared by mixing 68.4 ml of 1M Na_2HPO_4 and 31.6 ml of 1 M NaH_2PO_4. This was then made up to 1 L with Milli-Q water and autoclaved for 15 minutes at 121°C.

2.11.1.3 EDAC

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was stored at 4°C until use, at which time it was added with Affi-Gel 102 to a final concentration of 5 mg/ml, to the labelled β-lactam in order to terminate the labelling reaction.

2.11.1.4 Lysostaphin (1 mg/ml)

A 1 mg/ml solution of Lysostaphin (Sigma)was prepared by diluting the suspension of the enzyme received to a final concentration of 1 mg/ml. The enzyme was stored at -20°C until use, when it was thawed slowly to room temperature.

2.11.1.5 Ammonium Persulphate (APS)

A 10 % solution of APS was prepared by dissolving the appropriate amount of powder in Milli-Q water. Solutions were stored at 4°C and were made fresh every 2 to 3 days.

2.11.1.6 Upper Gel Buffer

A solution containing 0.5 M Tris-HCl (pH 6.8) and 10 % (w/v) SDS was made and the appropriate volume added to stacking gel mixtures.

2.11.1.7 Lower Gel Buffer

A solution containing 1.5 M Tris-HCl (pH 8.8) and 10 % (w/v) SDS was made and the appropriate volume added to separating gel mixtures.

2.11.1.8 Acrylamide-Bis Gels

Stacking gels: These gels had a final acrylamide-bis concentration of 4.5 % and contained (per 10 ml): Acrylamide/bis, 1.5 ml; Upper gel buffer, 2.5 ml; Milli-Q water, 6 ml; TEMED(Merck), 25 μl and APS (10 % w/v), 60 μl. Gels were made fresh prior to use

Separating gels: These gels had a final acrylamide/bis concentration of 10 % (Dargis and Malouin, 1994) and contained (per 10 ml): Acrylamide/bis, 3.3 ml; Lower gel Buffer, 4 ml; Milli-Q water, 2.7 ml, APS (10 % w/v), 50 μl and TEMED, 5 μl. Gels were prepared freshly before use and water saturated isobutanol was poured on their surface to ensure they were even.

2.11.1.9 Final Sample Buffer (FSB)

This solution contained (g/100 ml): Tris (pH 7.6), 0.76; SDS, 3; Bromophenol Blue, 0.01 and 10 ml glycerol. The solution was made up to 95 ml with Milli-Q water and stored at room temperature. β-mercaptoethanol was added to a final concentration of 5 % directly prior to use.

2.11.1.10 Bovine Serum Albumin (1 mg/ml)

A 1 mg/ml solution was prepared of the protein in Milli-Q water. This was mixed in equal volumes with final sample buffer and 10 μl run on a gel, giving 5 μg total protein, to provide a protein of known weight to help in the identification of PBP 2a.

2.11.1.11 Running Buffer

300 ml of a 5 x solution of the buffer contained (g/L): Tris, 15; Glycine, 72 and SDS, 5. The solution was made up to 300 ml with Milli-Q water. Prior to use, the buffer was diluted 5-fold in Milli-Q water to produce a 1x solution.

2.11.1.12 Transfer Buffer (pH 8.3)

The solution contained (g/L): Tris, 2.42; glycine, 14.41 dissolved in Milli-Q water. Concentrated hydrochloric acid was used to adjust the pH before the addition of methanol to give a final concentration of 4 %. Milli-Q water was added to make the final volume up to 1 litre.

2.11.1.13 Coomassie Brilliant Blue Stain

This solution contained (g/L): Coomassie Brilliant Blue, 2.5, dissolved in 454 ml methanol, 454 ml Milli-Q and 92 ml acetic acid to give a final volume of 1 L. The destaining solution was identical, with the omission of Coomassie Brilliant Blue. Gels were stained by gentle shaking in sufficient stain to cover their surface for 60 minutes or overnight. Destaining solution was poured over the gels in excess and the gels shaken. Destaining solution was replaced when it was no longer colourless and rinses repeated until the destaining solution no longer changed colour.

2.11.1.14 Tris Buffered Saline (TBS) (pH 7.6).

A solution containing 20 mM Tris and 13 mM NaCl contained (g/l): Tris, 2.42 and NaCl, 8.01. The solution was made up to 800 ml using Milli-Q water before the pH was adjusted to 7.6 using concentrated hydrochloric acid. The solution was then made up to a final volume of 1 litre with Milli-Q water.

2.11.1.15 Tween-20 Tris Buffered Saline (TTBS)

This solution was made by adding Tween-20 to TBS to a final concentration of 0.1 % (w/v).

2.11.1.16 Blocking Solution

This solution contained 3 % (w/v) skim milk powder dissolved in TTBS and was prepared prior to each Western Blot.

2.11.1.17 Streptavidin-peroxidase Conjugate

The conjugated streptavidin-horseradish peroxidase (Amersham) was diluted 10,000-fold in TTBS immediately before being added to the blot.

2.11.1.18 Amido Black

Amido Black destaining solution contained 25 % methanol and 10 % acetic acid, made up to 100 ml in Milli-Q water. The stain contained 0.075 g of Amido Black dissolved in 50 ml of the above solution.

2.11.2 Preparation of Samples

2.11.2.1 Preliminary Experiments to Identify PBP 2a

A 2 ml overnight culture of MRSA was diluted 1:50 to give two cultures with a final volume of 6 ml and incubated shaking at 37°C until the OD_{600} reached 0.4. After 15 minutes methicillin or ampicillin was added to induce PBP 2a production and 1 ml samples were removed after 15 minutes and then at 30 minute intervals until 90 minutes had passed and immediately frozen. Later experiments also included variations in concentrations of β-lactam antibiotic in order to try to induce the

production of PBP 2a, and in some cases were only sampled after 90 minutes as a check to see if induction had occurred. All experiments included MSSA as a negative control of PBP 2a production.

2.11.2.2 Determination of Effect of Totarol on PBP 2a Production

Cells were prepared as in the above description, with the modification that the test tube also contained totarol to a final concentration of 1 μg/ml, or half-MIC.

2.11.3 Labelling of β-lactam

The β-lactam being used to detect PBP 2a, either ampicillin or 6-aminopenicillanic acid was labelled by being mixed with a 5-fold molar excess of an N-hydroxysuccinamide ester of biotin (Pierce Chemicals) and dissolved in 0.1 M sodium phosphate buffer (Section 2.11.1.2). The solution was incubated at room temperature for 30 minutes with gentle agitation, after which time the reaction was stopped by the addition of an equal volume of Affi-Gel 102 (Bio-Rad) and 5 mg EDAC (Section 2.11.1.3.). The labelled β-lactam was then separated from the ligand by centrifugation at 14000 rpm for 2 minutes. Freshly labelled β-lactam was prepared before each experiment.

2.11.4 Labelling of PBP 2a

The 1 ml samples collected in Section 2.11.2 were spun down to form a pellet by centrifuging at 14000 rpm for 2 minutes before the broth was removed using a 1 ml pipette. Samples were resuspended in 50 μl PBS (pH 7.0) (Section 2.11.1.1) and labelled by the addition of the previously prepared β-lactam (Section 2.11.3). The labelled antibiotic was added to give a final concentration of 4 μg/ml and samples were incubated for 30 minutes at room temperature. 2 μl lysostaphin (Section 2.11.1.4) was added to lyse the samples which were then incubated at 37°C for 30 minutes or until samples were no longer turbid. Each sample was then boiled for 5 minutes in final sample buffer (Section 2.11.1.9) before the proteins were separated in duplicate gels by SDS-PAGE using stacking and separating gels containing 4.5 %

and 10 % acrylamide-bis respectively. In each gel 5 µl of molecular weight standards of known size (BRL) were run in one lane. An additional lane containing 10 µl bovine serum albumin (Section 2.11.1.10) was also included to provide a protein of similar weight to that of PBP 2a. All samples and standards were boiled in FSB in a 1:1 volume. A sufficient volume of running buffer (Section 2.11.1.3) was added to allow current to run through the gels which were then run for 90 to 120 minutes at 90 to 110 V.

2.11.5 Detection of PBP 2a

A Western Blot of one gel was produced by transferring proteins to a nitro-cellulose membrane at 4 V overnight or 90 minutes at 15 V in transfer buffer (Section 2.11.1.9.). The remaining acrylamide gel was stained in Coomassie Blue (Section 2.11.1.13) during the blotting procedure and destained to visualise the proteins in each sample by direct staining.

PBP 2a was detected using the method of Dargis and Malouin (1994). The following steps were used:

1. Wash blot by gentle agitation for 60 minutes in TTBS containing 3 % skim milk powder (Section 2.11.1.13) at room temperature
2. Rinse in TTBS (Section 2.11.1.12)
3. Wash in TTBS for 15 minutes at room temperature
4. Wash in TTBS for 5 minutes at room temperature, repeat
5. Bind streptavidin-peroxidase conjugate for 60 minutes at room temperature
6. Wash at 60°C in pre-heated TTBS for 15 minutes
7. Wash at 37°C in pre-heated TTBS for 15 minutes
8. Wash at 37°C in pre-heated TTBS for 5 minutes
9. Wash in TTBS at room temperature for 5 minutes, repeat twice
10. Mix equal volumes of ECL detection reagents and flood the blot after draining off excess TTBS.
11. Incubate at room temperature for 1 minute
12. Drain off excess reagents and wrap blot in cling film

13. Mark corners of the blot by making small asymmetric holes with a needle and immediately expose to X-ray film for 5 sec to 5 min depending on desired intensity of bands and develop.

2.11.6 Visualisation of Proteins on Blots Using Amido Black

In some instances Amido Black was used in order to be able to immediately visualise protein bands on blots. The blot was flooded with Amido Black (Section 2.11.1.15) and stained for 10 minutes before being washed with destaining solution until the background of the blot is clear.

3. RESULTS

3.1 Determination of MICs for Diterpene Derivatives

3.1.1 Determinations of MICs for Diterpene Derivatives with Methicillin-Sensitive *S. aureus*

The MICs and MBCs determined for each derivative with *S. aureus* strain 87 are presented in Table 3.1. The compounds with the lowest MICs (Section 2.5) were totarol (3.2 μg/ml), 258 (4 μg/ml), 390 and 416 (both 8 μg/ml). These derivatives were also found to have the lowest MBCs (Section 2.6).

3.1.2 Determination of MICs for Diterpene Derivatives with Methicillin-Resistant *S. aureus*

The MICs and MBCs determined for each diterpene with *S. aureus* strain MR96/164 are presented in Table 3.2. Again the derivatives with the lowest MICs were totarol (2 μg/ml), 258 (4 μg/ml), 416 (8 μg/ml) and 390 (16 μg/ml). These derivatives were also found to have the lowest MBCs.

3.1.3 Determination of MICs for Diterpene Derivatives with *E. coli*

E. coli strain C600 was used as a representative species to enable potentiation experiments to be carried out on a Gram negative bacterium. The MIC of each of the derivatives most effective against *H. pylori* were determined for *E. coli* strain C600, using the same method as for *S. aureus* (Section 2.5) and are listed in Table 3.3. PK16 had the lowest MIC at 32 μg/ml and all derivatives had MBCs of above 32 μg/ml.

Table 3.1 Determination of MIC and MBC for totarol and derivatives with MSSA strain 87

Derivative	MIC (μg/ml)	MBC (μg/ml)
totarol	3.2	3.2
211	32	32
254	16	32
258	4	8
278	32	>32
279	>32	>32
280	>32	>32
282	>32	>32
289	>32	>32
314	>32	>32
317	32	>32
390	8	32
391	>32	>32
416	8	16
PK1	>32	>32
PK2	>32	>32
PK3	32	>32
PK4	32	>32
PK5	>32	>32
PK6	>32	>32
PK7	>32	>32
PK8	>32	>32
PK9	>32	>32
PK10	>32	>32
PK11	32	32
PK12	32	>32
PK13	>32	>32
PK14	32	>32
PK15	16	32
PK16	32	>32

Table 3.2 Determination of MIC and MBC for totarol and derivatives for MRSA strain MR96/164

Derivative	MIC (μg/ml)	MBC (μg/ml)
totarol	2	2
podocarpic acid	>32	>32
211	32	32
254	32	32
258	4	16
278	>32	>32
279	32	>32
280	>32	>32
282	>32	>32
289	>32	>32
314	>32	>32
317	32	>32
390	16	32
391	>32	>32
416	8	8
PK1	>32	>32
PK2	>32	>32
PK3	>32	>32
PK4	>32	>32
PK5	>32	>32
PK6	>32	>32
PK7	32	32
PK8	32	>32
PK9	32	>32
PK10	32	>32
PK11	32	>32
PK12	32	>32
PK13	>32	>32
PK14	32	>32
PK15	32	>32
PK16	32	>32

Table 3.3 Determination of MIC and MBC for derivatives with greatest activity against *H. pylori* with *E. coli* C600

Derivative	MIC (μg/ml)	MBC (μg/ml)
289	>32	>32
PK2	>32	>32
PK16	32	>32

3.2 Determination of MIC of Antibiotics with MRSA

The MICs for each of the β-lactam antibiotics tested with MRSA, as determined by standard macrobroth dilution (Section 2.5) are presented in Table 3.4. Methicillin was selected as the antibiotic to be used in subsequent experiments. Strain 87 was found to be sensitive to all the β-lactams tested to a concentration of 2 μg/ml or less as expected (not shown).

3.3 Disk Diffusion Tests for Diterpenes with *H. pylori*

The results of the disk diffusion tests (Section 2.7) for each of the 30 derivatives tested are presented in Table 3.5. The derivative showing the greatest efficacy was PK16, which had the largest zone of inhibition (22 mm). This compound also showed inhibition of *H. pylori* when only 1 μg final amount was present on the antibiotic disk. The other two derivatives that showed inhibition at this level were 289 and PK2. The majority of derivatives (53%) showed some inhibition of growth when 5 μg of derivative was present; all other derivatives required 25 μg or more to inhibit the growth of *H. pylori*.

3.4 Potentiation of β-lactam Activity

3.4.1 Potentiation of Methicillin Activity Against MRSA

The MIC and MBC for methicillin in the presence of 10 μg/ml or half-MIC of totarol and each derivative with MRSA strain MR96/164 is shown in Table 3.6. The MIC for methicillin with strain MR96/164 had been previously determined to be 32 μg/ml. This antibiotic was used in the potentiation experiments as it was the β-lactam antibiotic with the lowest independent MIC other than imipenem, to which the strain was determined to be sensitive.

Table 3.4 Determination of MIC of β-lactam antibiotics with MRSA strain MR96/164

β-lactam	MIC (μg/ml)
Methicillin	32
Ampicillin	>128
Penicillin-G	>2048
Imipenem	2

Table 3.5 Zones of inhibition for totarol and derivatives with *H. pylori*

Derivative	Zone of Inhibition (mm)					MIC (μg)
	0μg	1μg	5μg	25μg	50μg	
totarol	6	6	6.5	7	8	5
211	6	6	7	10	12	5
254	6	6	6	6	6	>50
258	6	6	6	6	6	>50
278	6	6	6	7	8	25
279	6	6	6	6	6	>50
280	6	6	6	6	6	>50
282	6	6	7	11	14	5
289	6	8	9	12	13	1
314	6	6	6	6	6	>50
317	6	6	7	10	11	5
390	6	6	6	7	11	25
391	6	6	7	9	10	5
416	6	6	7	9	10	5
PK1	6	6	7	8	9	5
PK2	6	7	7.5	8	10	1
PK3	6	6	7	8	10	5
PK4	6	6	7	12	14	5
PK5	6	6	7	10	14	5
PK6	6	6	6	6	6	>50
PK7	6	6	8	11	14.5	5
PK8	6	6	7	9	10	5
PK9	6	6	7	10	12	5
PK10	6	6	6.5	7	9	5
PK11	6	6	7	9	10	5
PK12	6	6	8	10	9	5
PK13	6	6	6	13	14	25
PK14	6	6	7	9	10	25
PK15	6	6	7	9	10	25
PK16	6	6.5	7	12	22	1

note: the diameter of the disk is 6 mm and zones of diameter were measured across the disk, as described in the Manual of Clinical Microbiology (6th Edition).

Table 3.6 Determination of MIC of methicillin for MRSA strain MR96/164 in the presence of 10μg/ml or half-MIC diterpene

Derivative	MIC (μg/ml)	MBC (μg/ml)
totarol	4	>32
podocarpic acid	32	>32
211	32	>32
254	32	>32
258	8	>32
278	>32	>32
279	32	>32
280	>32	>32
282	32	>32
289	>32	>32
314	>32	>32
317	32	>32
390	<2	>32
391	>32	>32
416	<0.125	>32
PK1	>32	>32
PK2	32	>32
PK3	32	>32
PK4	>32	>32
PK5	>32	>32
PK6	32	>32
PK7	16	>32
PK8	32	>32
PK9	32	>32
PK10	16	>32
PK11	32	>32
PK12	8	>32
PK13	32	>32
PK14	8	>32
PK15	4	>32
PK16	32	>32

The results for these experiments are expressed as an MIC for methicillin with the strain, grown in the presence of diterpene derivative. The compounds showing the greatest potentiation activity, as determined by reducing the MIC for methicillin to the greatest extent, were 258, PK12 and PK14 (8 μg/ml), totarol and PK15 (4 μg/ml), 390 (<2 μg/ml) and 416 (<0.125 μg/ml). This range of derivatives included all those found to possess the greatest independent activity. The MBC was greater than 32 μg/ml for all the compounds tested.

3.4.2 Potentiation of Methicillin Activity Against *E. coli*

The MIC of methicillin for *E. coli* strain C600 was determined to be greater than 32 μg/ml by standard macrobroth dilution (Section 2.5), as for other MIC determinations. The potentiation activity of those derivatives showing the greatest activity against *H. pylori* was investigated by determining the MIC for methicillin in the presence of 10 μg/ml of each of these derivatives (Section 2.8) and the results are presented in Table 3.7. The MIC for each derivative with *E. coli* had been previously determined and in each case was greater than 10 μg/ml, so this concentration was used. The observed methicillin MIC in the presence of each of the derivatives tested was determined to be greater than 32 μg/ml, indicating none of the diterpene derivatives tested possessed potentiation activity against a Gram negative bacterium.

3.5 Effect of Totarol on DNA Synthesis

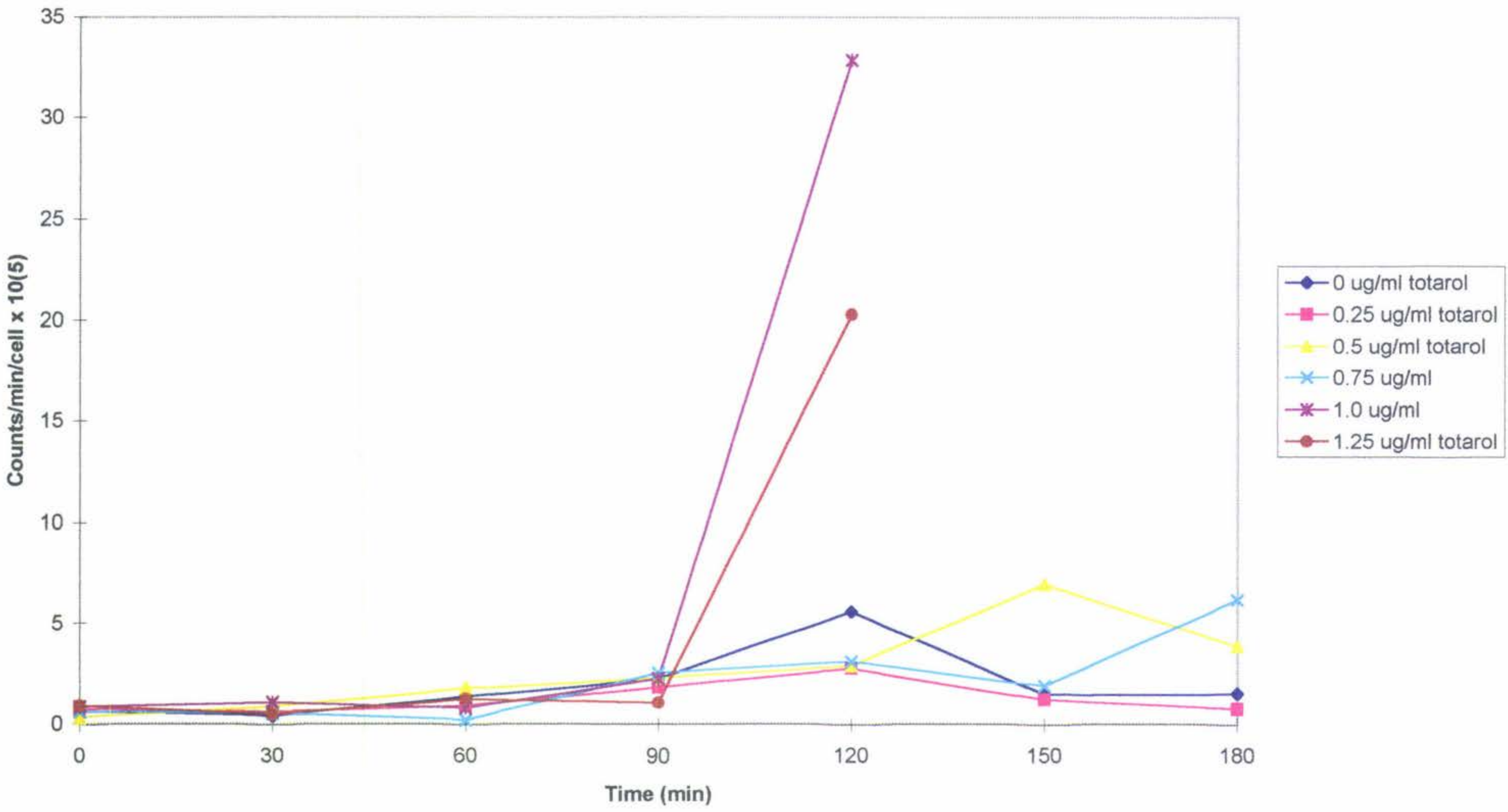
3.5.1 Measurement of the Rate of Thymidine Incorporation in the presence of Totarol by Viable Cell Determination

The rate of incorporation of tritiated methyl-thymidine (Section 2.9.2.2) into the DNA of MRSA under varying concentrations of totarol was measured in three ways. The first method was to measure incorporation as a function of viable cells (Section 2.9.2.5, part 1). Typical data is presented in Figure 3.1.

Table 3.7 Determination of MIC and MBC of methicillin for *E. coli* C600 in the presence of 10μg/ml diterpene derivative

Derivative	MIC (μg/ml)	MBC (μg/ml)
289	>32	>32
PK2	>32	>32
PK16	>32	>32

Figure 3.1 Thymidine incorporation per viable cell by MRSA strain MR96/164 in the presence of increasing concentrations of totarol.


35
30
25
20
15
10
5
0
Counts/min/cell x 10(5)
0
30
60
90
120
150
180
Time (min)
0 ug/ml totarol
0.25 ug/ml totarol
0.5 ug/ml totarol
0.75 ug/ml
1.0 ug/ml
1.25 ug/ml totarol

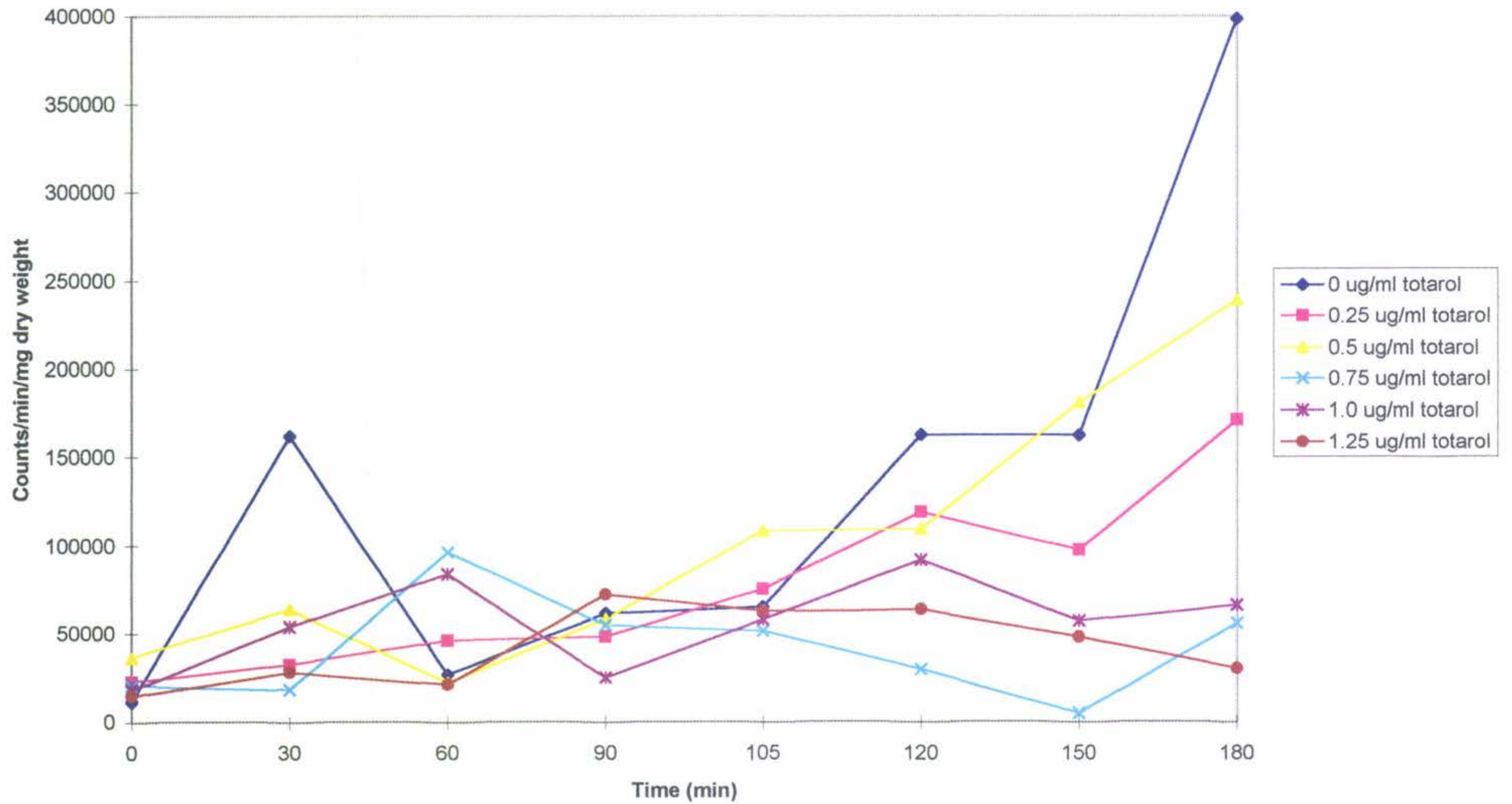
In each experiment there was markedly higher incorporation per cell in the higher concentrations of totarol. In the lower concentrations of totarol and the control, incorporation per viable cell occurred at a reasonably steady rate, while in the two highest concentrations of totarol there was typically a very high rate of incorporation per cell. This would indicate that inhibition of DNA synthesis is not the mode of action of totarol, as DNA synthesis is still occurring. Instead this would indicate that the mode of action is another cellular target and that although the cells are being killed by totarol, the diterpene is acting on another target, allowing DNA synthesis to continue until cell death.

3.5.2 Measurement of the Rate of Thymidine Incorporation in the Presence of Totarol by Determination of the Dry Weight of Cultures

The second method by which the effect of totarol on DNA synthesis was measured was by determination of the rate of incorporation of labelled thymidine per microgram dry weight of culture (Section 2.9.2.5, part 2) Initially 0.1 ml samples were removed for dry weight determination. However, this proved to yield too small a weight to measure accurately and it was impossible to gain an accurate determination of the dry weight of the culture.

The volume sampled for dry weight determination was increased to 0.5 ml in an attempt to sample sufficient cells for accurate weight measurement. Typical data obtained in this experiment is shown in Figure 3.2. Again the resulting dry weights of samples were very small, ranging from 0.3 to 1.5 mg. This was at the very smallest level that could be read on the balance and as such some error may have occurred as no relationship between totarol concentration and dry weight was discernible. Although the scintillation counts showed a decrease with increasing totarol concentration, the trend was not so clear when incorporation was expressed relative to micrograms of dry weight. The overall result did however seem to indicate that there was no significant inhibition of DNA synthesis in the presence of totarol, as even in the highest concentrations of totarol there was incorporation.

Figure 3.2 Thymidine incorporation per milligram dry weight for MRSA strain MR96/164 in the presence of increasing concentrations of totarol.



400000
350000
300000
250000
200000
150000
100000
50000
0
Counts/min/mg dry weight
0
30
60
90
105
120
150
180
Time (min)
0 ug/ml totarol
0.25 ug/ml totarol
0.5 ug/ml totarol
0.75 ug/ml totarol
1.0 ug/ml totarol
1.25 ug/ml totarol

It became apparent that larger volumes would need to be sampled in order to measure the dry weights of the culture. However, financial limitations on the amount of isotope that could be purchased prevented the use of larger culture volumes, so a further method of measuring the growth of cultures was adopted.

3.5.3 Measurement of the Rate of Thymidine Incorporation in the Presence of Totarol by Determination of Total Cellular Protein

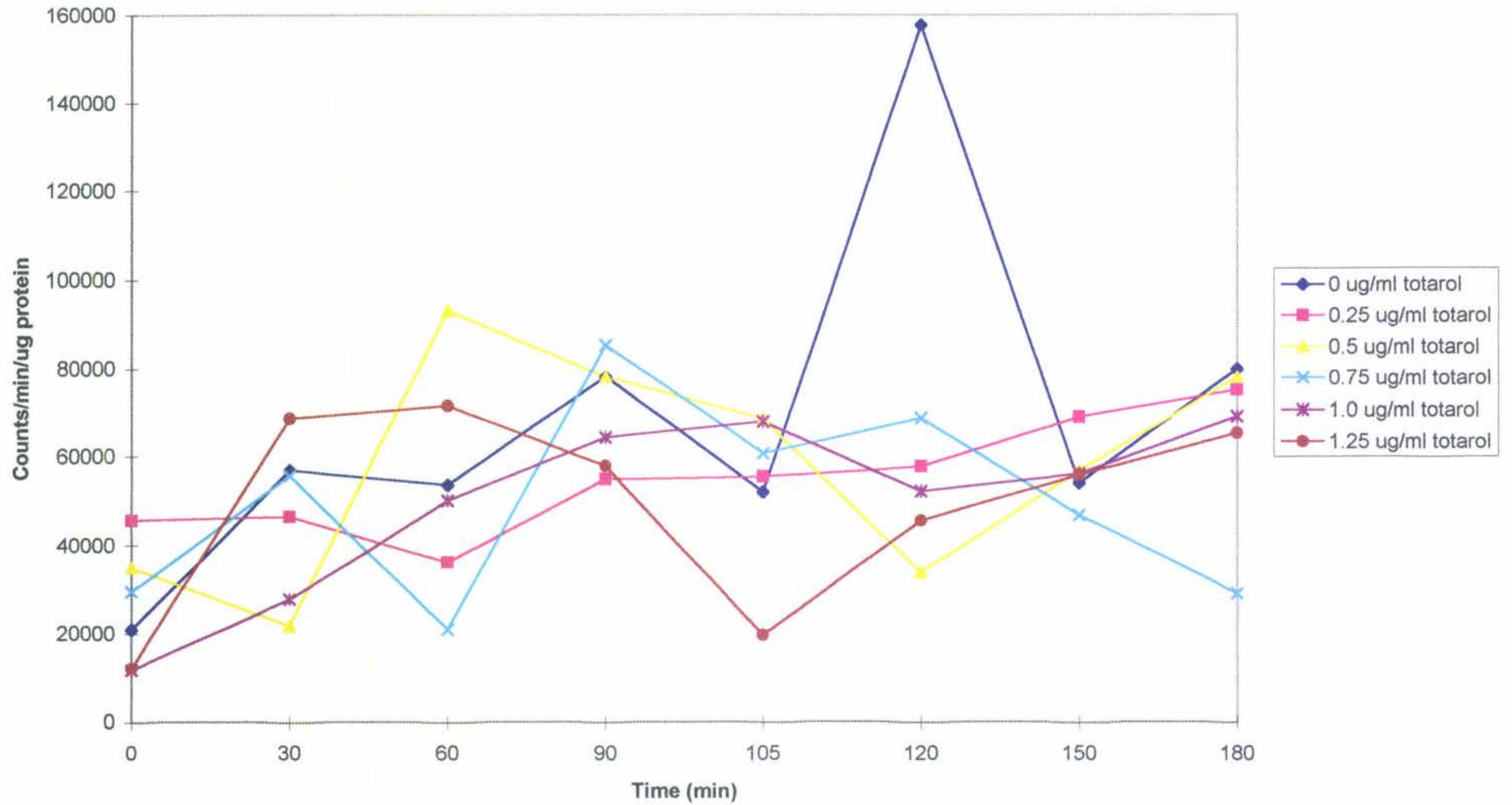
A protein assay (Bio-Rad, Section 2.9.2.5, part 3) was utilised to determine the total protein in each sample, thereby giving an indication of the growth of each culture. Initially the microassay procedure for microtitre plates was used, which produced a standard curve linear from 8 to 80 μg protein/ml. However, once again the samples proved to be too low for the assay, with many samples falling below the linear part of the standard curve, and readings therefore were not accurate. The standard microassay was instead adopted, producing a standard curve linear from 1 to 10 μg protein/ml which proved to be appropriate for the conditions of the experiment.

Typical data generated by this experiment is presented in Figure 3.3. Although one point on the graph is unusually high and is probably an artefact of the protein assay, all the cultures appeared to be incorporating totarol at around the same rate per microgram protein. This would indicate that totarol does not significantly inhibit DNA synthesis, a result also seen in the previous two methods of culture growth determination.

3.6 Investigation of the Effect of Totarol on Protein Synthesis

Two methods were used to determine the effect of totarol on protein synthesis in MRSA. The determination as a function of dry weight was omitted due to the reasons discussed above. The methods used in the determination of the effects of totarol on protein synthesis were viable cell count and determination of the total protein.

Figure 3.3 Thymidine incorporation per microgram protein by MRSA strain MR96/164 in the presence of increasing concentrations of totarol.

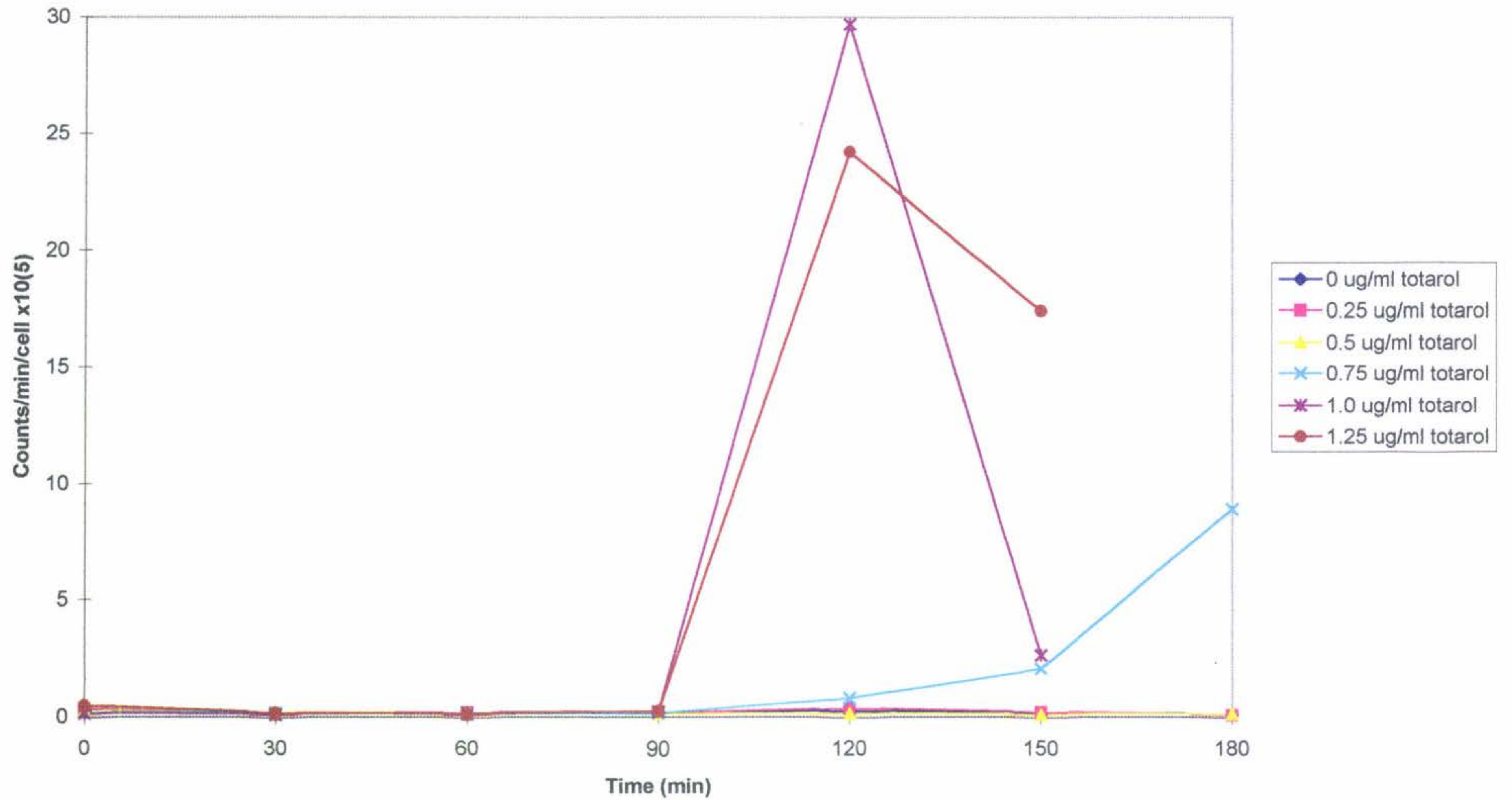


160000
140000
120000
100000
80000
60000
40000
20000
0
Counts/min/ug protein
0
30
60
90
105
120
150
180
Time (min)
0 ug/ml totarol
0.25 ug/ml totarol
0.5 ug/ml totarol
0.75 ug/ml totarol
1.0 ug/ml totarol
1.25 ug/ml totarol

3.6.1 Measurement of the Rate of Glutamine Incorporation in the Presence of Totarol by Viable Cell Determination

The rate of incorporation of tritiated glutamine (Section 2.9.3) was determined per viable cell for each of the concentrations of totarol in the same way as for thymidine (Section 2.9.2.2) using the precipitation method described in Section 2.9.3. Figure 3.4 presents typical data generated in such an experiment in which a similar trend to that observed in the incorporation of thymidine was seen with very high rates of incorporation per cell at the two highest concentrations of totarol, 1.0 and 1.25 μg/ml. As with the thymidine experiments, the rates of incorporation in the other cultures were at a similar rate, and it was interpreted that totarol did not cause significant inhibition of protein synthesis. Incorporation was still occurring at the highest concentrations of totarol, indicating the protein synthesis mechanisms of the cell were still functioning and suggesting that totarol must kill the cells by attacking some other primary target.

Figure 3.4 Glutamine incorporation per viable cell by MRSA strain MR96/164 in the presence of increasing concentrations of totarol.


30
25
20
15
10
5
0
Counts/min/cell x10(5)
0
30
60
90
120
150
180
Time (min)
0 ug/ml totarol
0.25 ug/ml totarol
0.5 ug/ml totarol
0.75 ug/ml totarol
1.0 ug/ml totarol
1.25 ug/ml totarol

3.6.2 Measurement of the Rate of Glutamine Incorporation in the Presence of Totarol by Determination of Total Protein

It was determined during the experiments investigating the incorporation of thymidine that the standard microassay procedure for total protein determination was the most suitable for the conditions of the experiment and as a result was the method used to determine total protein in these experiments.

Typical data is presented in Figure 3.5. As with the thymidine incorporations, there was incorporation in all totarol concentrations to approximately the same levels, indicating that the protein synthesis mechanisms were intact in the presence of totarol. This would again suggest that inhibition of protein synthesis is not the mode of action of totarol, and that the diterpene must act against a different cellular target. As with the thymidine incorporation experiments, it was anticipated that inhibition of protein synthesis would be apparent by a much lower rate of glutamine incorporation and hence protein synthesis in the higher concentrations of totarol.

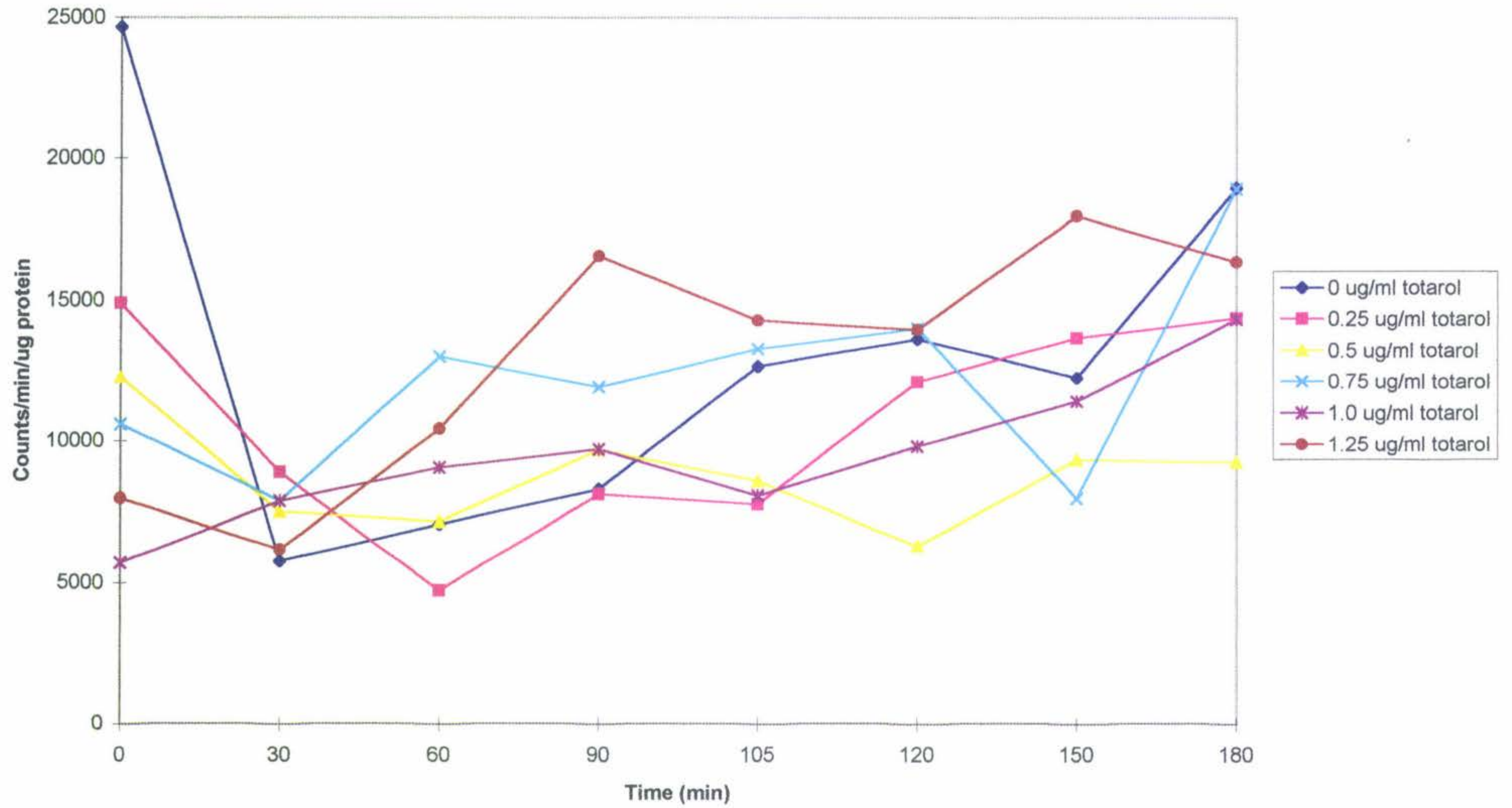
3.7 Investigation of the Effect of Totarol on Peptidoglycan Synthesis

3.7.1 Optimisation of Recovery of Labelled Peptidoglycan

Tritiated N-acetyl glucosamine (Section 2.9.4) was the precursor of peptidoglycan used to measure the rate of synthesis of the molecule.

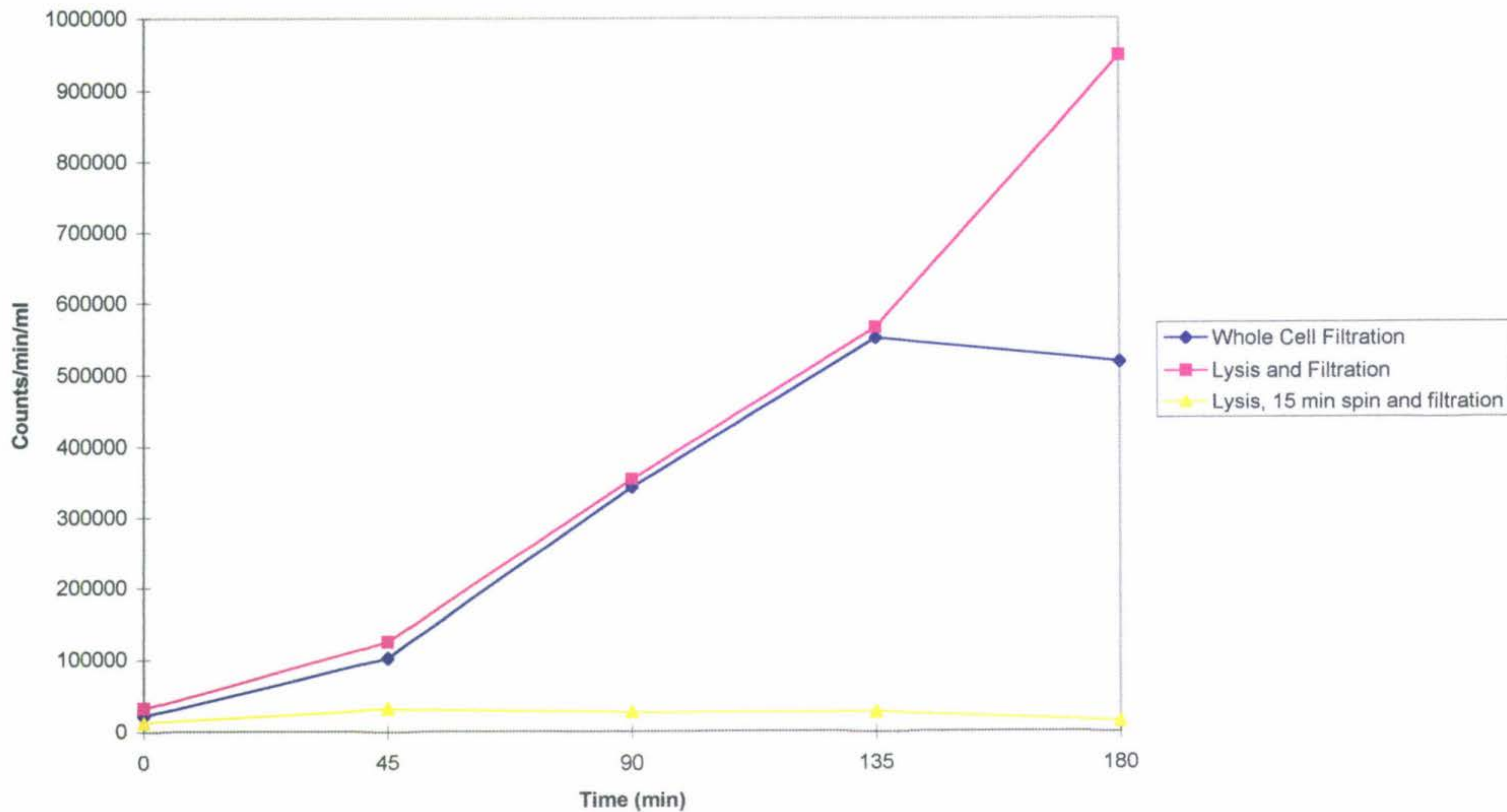
Three methods were utilised to first isolate the labelled peptidoglycan (Section 2.9.4); filtration of whole cells, filtration of lysed cells and filtration of resuspended pellet from lysed cells. Typical data generated from the comparison of the three methods is depicted in Figure 3.6. The most efficient method was determined to be the filtration of lysed

Figure 3.5 Glutamine incorporation per microgram protein by MRSA strain MR96/164 in the presence of increasing concentrations of totarol.



25000
20000
15000
10000
5000
0
Counts/min/ug protein
0
30
60
90
105
120
150
180
Time (min)
0 ug/ml totarol
0.25 ug/ml totarol
0.5 ug/ml totarol
0.75 ug/ml totarol
1.0 ug/ml totarol
1.25 ug/ml totarol

Figure 3.6 Total incorporation of N-acetyl glucosamine by MRSA strain MR96/164 after various peptidoglycan isolation procedures.



1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Counts/min/ml
0
45
90
135
180
Time (min)
Whole Cell Filtration
Lysis and Filtration
Lysis, 15 min spin and filtration

cells, as can be seen by the high scintillation counts for this procedure. This preparation of cells was used for all subsequent peptidoglycan incorporation experiments.

3.7.2 Confirmation of Measurement of Peptidoglycan Synthesis

A control experiment was conducted to ensure that N-acetyl glucosamine incorporation (Section 2.9.4) was an accurate indication of the rate of peptidoglycan synthesis. Data from this is presented in Figure 3.7 and showed much greater incorporation in the control tube. The test culture containing 1 μg/ml (half-MIC) vancomycin showed very little incorporation, around 50,000 counts/min/ml over the course of the 180 minutes the experiment was conducted while incorporation increased with time in the control tube up to almost 500,000 counts/min/ml at 180 minutes, a 10-fold increase on the incorporation in the test culture. This clearly showed both that the level of vancomycin used inhibited peptidoglycan synthesis, and also that the rate of incorporation of N-acetyl glucosamine was a good indication of peptidoglycan synthesis.

3.7.3 Measurement of N-acetyl Glucosamine Incorporation in the Presence of Totarol by Determination of Total Protein

The protein assay method (Section 2..9.2.5, part 3) of determining the growth of the culture was used in the measurement of incorporation of N-acetyl glucosamine in the presence of totarol by MRSA as it had been previously determined to be the most appropriate. Typical data is shown in Figure 3.8 and again shows no significant decrease in the rate of incorporation of N-acetyl glucosamine with increasing concentrations of totarol. As with the previous two incorporation experiments it was anticipated that an inhibitory effect on peptidoglycan synthesis would be indicated by lower rates of incorporation at the higher totarol concentrations. However, the rate of incorporation per microgram protein is similar across all concentrations of totarol, indicating that peptidoglycan synthesis is not significantly inhibited by totarol. As in

Figure 3.7 Total incorporation of N-acetyl glucosamine by MRSA strain MR96/64 in the presence and absence of 0.5x MIC (1 μg/ml) vancomycin.

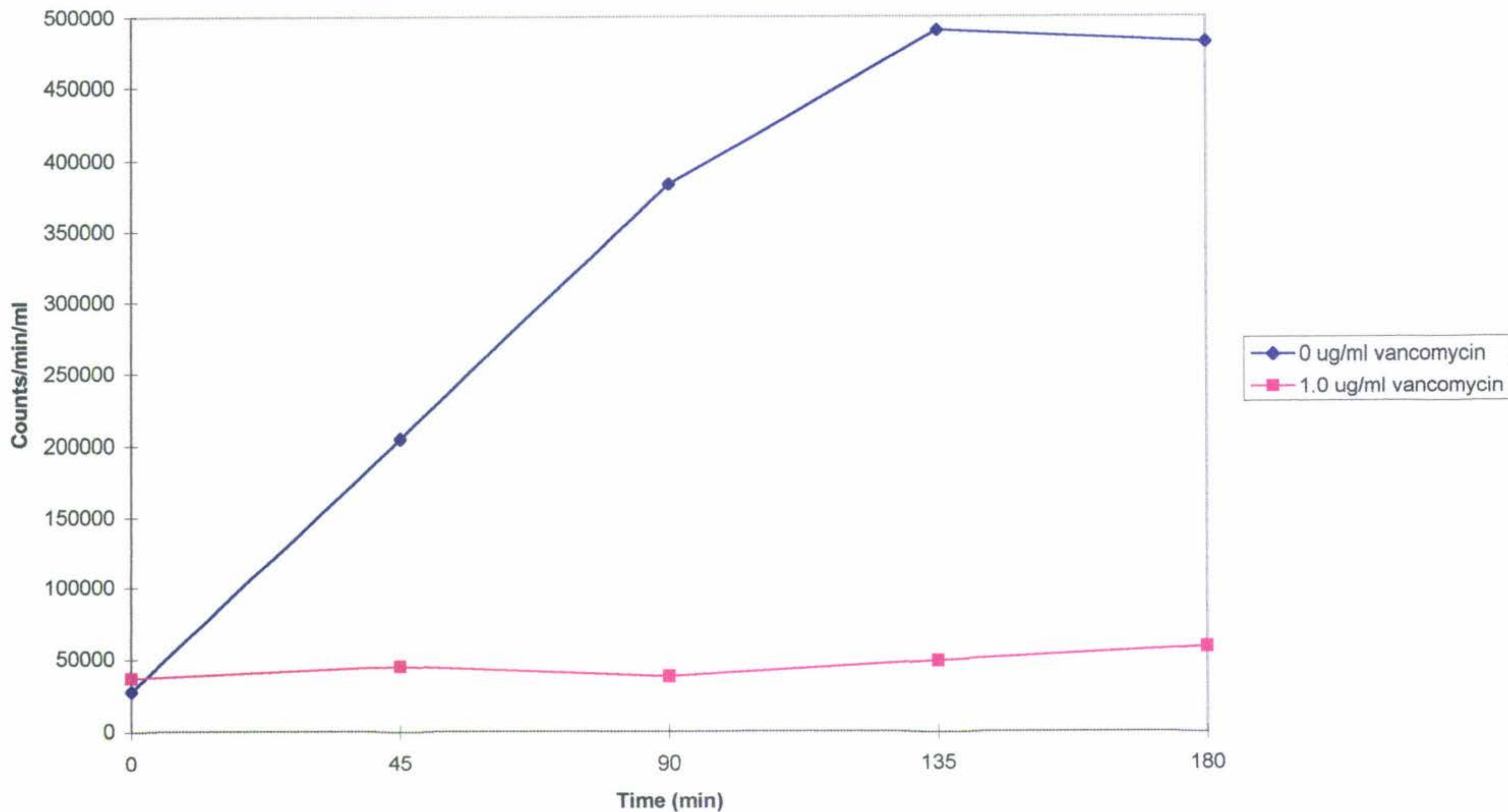
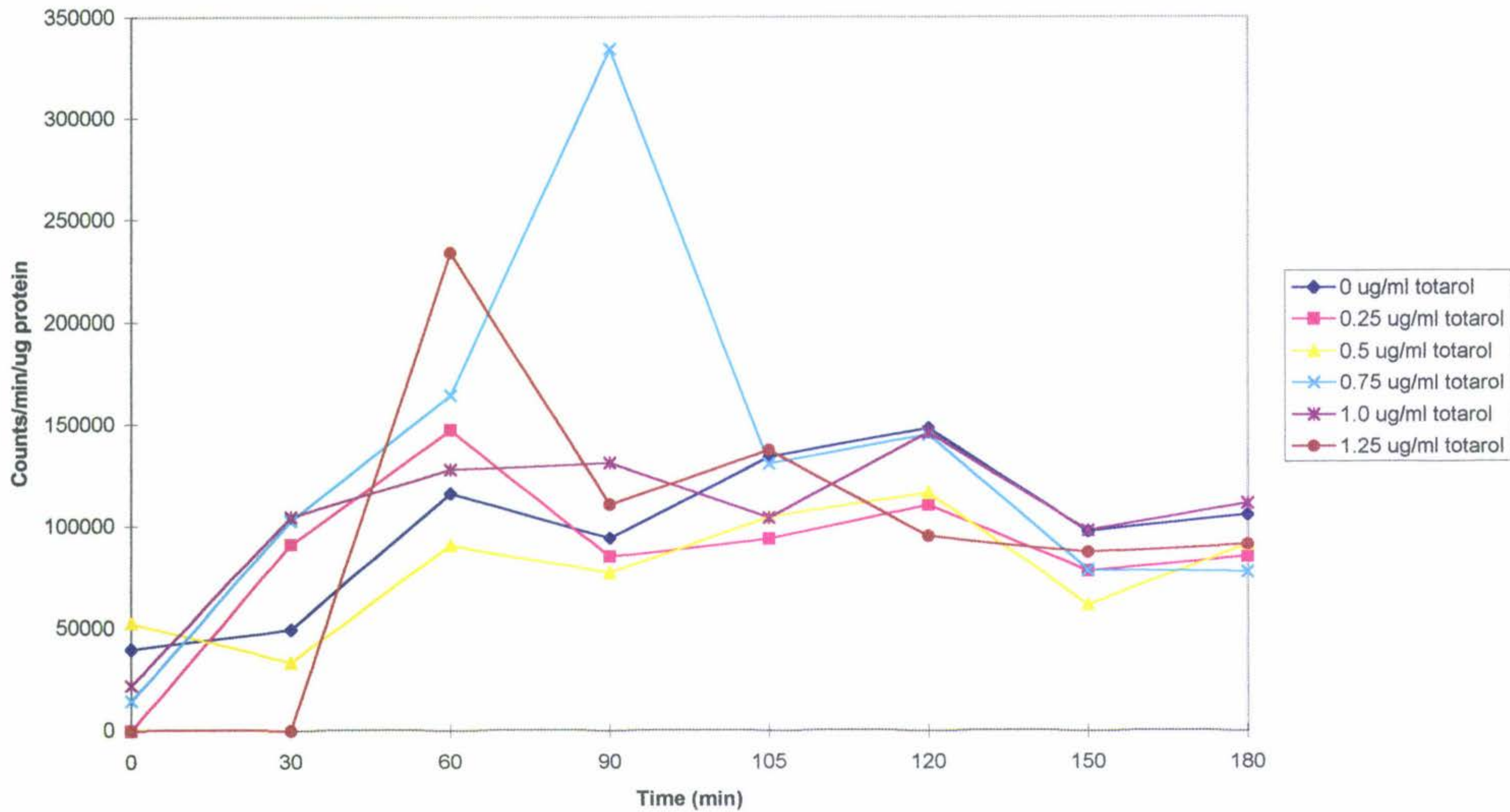

500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
Counts/min/ml
0
45
90
135
180
Time (min)
0 ug/ml vancomycin
1.0 ug/ml vancomycin

Figure 3.8 Incorporation of N-acetyl glucosamine per microgram protein by MRSA strain MR96/164 in the presence of increasing concentrations of totarol.


350000
300000
250000
200000
150000
100000
50000
0
Counts/min/ug protein
0
30
60
90
105
120
150
180
Time (min)
0 ug/ml totarol
0.25 ug/ml totarol
0.5 ug/ml totarol
0.75 ug/ml totarol
1.0 ug/ml totarol
1.25 ug/ml totarol

the previous two incorporation experiments, the expression of incorporation per microgram protein allowed the direct comparison of scintillation counts seen in all totarol concentrations, despite the lower number of viable cells in the higher concentrations of totarol.

3.8 Effect of Totarol on Cellular Respiration

In a preliminary experiment, 0.5 ml of cells were placed in the chamber with 4.5 ml of the glucose/salt solution used for these experiments (Section 2.10.1.1) and after 3 minutes of temperature equilibration, the rate of respiration was measured by observing 10 minutes of a steady rate of oxygen depletion. This proved to result in a steady rate of oxygen consumption. However, it had been suggested (Dr Jan Schmid, personal communication), that using a sample of bacteria in broth could exert some background effects on oxygen depletion due to the broth. A control experiment was conducted using 0.5 ml of LB (Section 2.2.3) in 4.5 ml glucose/salt solution. There was no decrease in the oxygen concentration of the solution which remained steady over a 30 minute period. It was concluded that the respiration could then be measured using 0.5 ml samples of cells in 4.5 ml glucose/salt solution.

Seven concentrations of totarol were tested, ranging from 3.5×10^{-2} (5 x MIC) to 7×10^{-5} mM (0.01 x MIC). Dilutions of totarol were prepared (Section 2.3) to allow the same volume of totarol, 17.5 μl, to be added to the chamber to prepare each final concentration. Although respiration had previously been measured using 10 minute stages for the experiment, 5 minutes only was allowed for the measurement of respiration under control conditions, as it was found that the oxygen level of the solution became too low using 10 minutes for each stage of the experiment, and it was difficult to measure the decrease in oxygen concentration over the final minutes of the experiment. By reducing the length of time required for each experiment it was determined that the concentration of oxygen in the chamber remained sufficiently high to retain accurate measurement of its depletion.

The results of testing the range of totarol concentrations described above are presented in Table 3.8 and shown in Figure 3.9. It was determined that there was inhibition of respiration at high concentrations of totarol, from 3.5×10^{-3} mM (0.5 x MIC) and there was complete inhibition of respiration at 3.5×10^{-2} mM (5 x MIC). This precluded the measurement of the effect of totarol at 0.1 mM which had also been intended as it was a concentration used by Haraguchi *et al*. (1996) determined to inhibit respiration in *S. aureus.*

At lower concentrations, it was found that totarol slightly increased the respiration rate of MRSA strain MR96/164. This was investigated further by adding 17.5 μl of methanol only to the chamber. It was found that the addition of this volume of methanol caused an increase in respiration to the same degree as that observed with the lower concentrations of totarol.

Due to time constraints, there was not sufficient time to investigate the effects of totarol and the other derivatives on respiration under potentiation conditions as had also been intended.

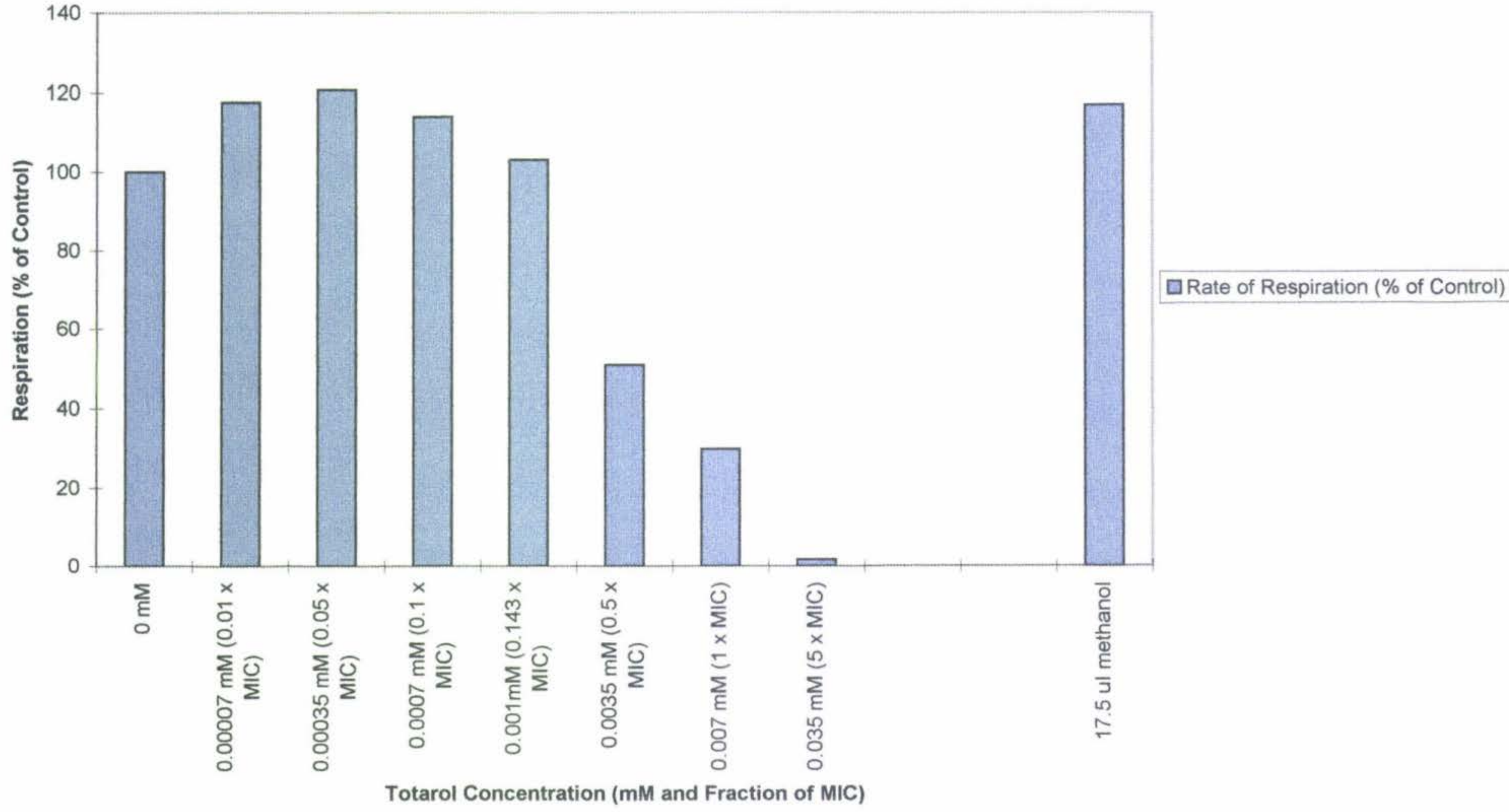
3.9 Interaction of Totarol with PBP 2a

Preliminary data from other groups has suggested that totarol may potentiate the activity of β-lactam antibiotics by inhibiting the expression or function of PBP 2a, the main resistance mechanism in MRSA (Chamberland *et al*., 1995). This activity may be important in the potential use of totarol as an adjunct for existing antibiotics. The aim of this section of research was to determine if this was the mode of action of totarol in potentiation conditions.

Table 3.8 Effect of totarol on respiration of MRSA

Totarol concentration (mM and Fraction of MIC)	Rate of Respiration Before Addition of Totarol (μl O_2/min/mg dry weight)	Rate of Respiration After Addition of Totarol (μl O_2/min/mg dry weight)	Change in Respiration Rate (% of Control)
3.5×10^{-2} (5 x MIC)	0.746	0.013	1.742
7×10^{-3} (1 x MIC)	0.83	0.249	30
3.5×10^{-3} (0.5 x MIC)	0.829	0.423	51.02
1×10^{-3} (0.143 x MIC)	0.882	0.909	103.06
7×10^{-4} (0.1 x MIC)	0.812	0.928	114.29
3.5×10^{-4} (0.05 x MIC)	0.832	1.005	120.79
7×10^{-5} (0.01 x MIC)	0.842	0.99	117.58
17.5 μl methanol	0.98	1.148	117.14

Figure 3.9 Respiration of MRSA strain MR96/164 in the presence of increasing concentrations of totarol.


Respiration (% of Control)
140
120
100
80
60
40
20
0
0 mM
0.00007 mM (0.01 x MIC)
0.00035 mM (0.05 x MIC)
0.0007 mM (0.1 x MIC)
0.001mM (0.143 x MIC)
0.0035 mM (0.5 x MIC)
0.007 mM (1 x MIC)
0.035 mM (5 x MIC)
17.5 ul methanol
Totarol Concentration (mM and Fraction of MIC)
Rate of Respiration (% of Control)

3.9.1 Preliminary Experiment to Determine if PBP 2a Could be Visualised by Direct Staining.

Preliminary experiments were conducted to determine whether there were any obvious differences between cultures in the presence of totarol and those without totarol, that is to determine whether PBP 2a could be visualised on a protein gel by direct protein staining. Totarol was added to each of five MRSA cultures to final concentrations of 0, 0.2, 0.5, 1.0 and 1.5 μg/ml. Samples were prepared as described in Section 2.11.4, electrophoresed in acrylamide gels (Section 2.11.1.3) and the protein visualised by staining with Coomassie Blue (Section 2.11.1.8). PBP 2a has a molecular weight of 78 kilodaltons (kDa) (Chambers, 1988) and so would be expected to appear between the second and third bands in the molecular weight standard (BRL) which correspond to 107.37 and 68.375 kDa. However, it was not possible to visualise any proteins in control samples that were not detectable in the presence of totarol. Future experiments were conducted using the detection method described in Section 2.11.5.

3.9.2 Results of Detection of PBP 2a Using Biotinylated β-Lactams

Nine strategies were employed in order to induce, detect and visualise PBP 2a. In initial experiments methicillin was added 15 minutes after the experiment had started to induce the protein and ampicillin bound to biotin (Bio-Amp, Section 2.11.3) was used to label the protein in samples. It was decided that totarol would not be added to samples until the protein had been successfully detected on blots and could easily be identified. After this time experiments would be conducted with totarol added at the initiation of the experiment and blots inspected to determine whether the band attributed to PBP 2a was absent in samples grown in the presence of totarol.

A test culture was prepared with methicillin added to a final concentration of 4 μg/ml and a control sample with no antibiotic. Samples were taken at 0, 15, 30, 60 and 90 minutes after the cultures had reached OD_{600} of 0.4. Samples were processed and the

Western Blot conducted as described in Section 2.11.5. However, no band was detected that could be attributed to PBP 2a.

A methicillin titration was incorporated into the following experiment to investigate whether the lack of PBP 2a was due to induction of the protein not being stimulated by the concentration of methicillin previously used. Six parallel cultures were prepared, and methicillin added after 15 minutes as described previously, to give final concentrations of 4, 8, 16, 32, 64 and a control containing 0 μg/ml methicillin. It was anticipated that PBP 2a would be detected in those concentrations of methicillin causing full induction and therefore expression of the protein. The range of methicillin concentrations tested were sufficiently high to include the MIC and above, as well as lower, non-inhibitory concentrations. The stock solution of methicillin was also tested (Section 2.5) to ensure it was still fully active. However, no clear induction of PBP 2a was evident with bands appearing in all sample lanes, none of which corresponded in position to that which would be expected for PBP 2a. There was no apparent pattern in intensity of bands with increasing antibiotic concentration.

Other strategies were then employed, including labelling samples for a longer period of time than that described by Dargis and Malouin (1994). Samples were labelled with freshly prepared label for 60 minutes instead of 30. There was still no identifiable PBP 2a band. The dilution of streptavidin-peroxidase conjugate (Section 2.11.1.14) was also investigated to ensure it was not being used at a higher concentration than that recommended by the manufacturers, which could result in less specific binding patterns. However, the 10,000 fold dilution used in this experiment was ten times greater than that recommended by the manufacturers which should have resulted in a more specific binding reaction. Samples containing the supernatant of the lysed cells (Section 2.11.4) were also loaded on gels instead of the pellet in order to investigate the possible loss of PBP 2a with the supernatant. Samples were also tested that had been prepared by omitting the centrifugation step to investigate whether the protein was being lost at this point. It was still not possible to positively identify PBP 2a.

Samples of MSSA cells were also included in gels to provide a non-PBP 2a-producing control to compare results with. MSSA cultures were treated in exactly the same way as MRSA samples and run on gels. Samples that were both induced with methicillin and uninduced were run together. There were no differences between cultures with the same bands appearing in both cultures. The induction of PBP 2a was also attempted using penicillin in the place of methicillin. MRSA and MSSA cultures, both induced and uninduced by both antibiotics were run on a gel together and blotted, without the identification of PBP 2a. The result of a typical blot depicting this is shown in Figure 3.10. In many blots such as this, there were no bands visible other than an apparently non-specific band that appeared in all blots and was present in all samples.

An alternative antibiotic for the labelling of PBP 2a was also used. Dargis and Malouin (1994) also labelled their protein with 6-aminopenicillanic acid in their detection of PBP 2a so this was also attempted. A Western Blot was conducted with samples of MRSA and MSSA grown in the presence of 0, 32 and 100 μg/ml methicillin. In this case a band was detected only in MRSA cells that had been treated with antibiotic (Figure 3.11). However, its position on the gel relative to the molecular weight standard did not correspond to the position which would be expected for PBP 2a and the experiment was not repeated.

In another experiment, there were no distinct bands of the correct size in any samples and the only bands present in MRSA lanes only were of a molecular weight of 50 kDa or less; much smaller than that of PBP 2a, which has a molecular weight of 78 kDa. These bands had been observed in samples labelled with 6-aminopenicillanic acid, so it was decided to repeat the experiment using ampicillin to label the cells. All controls were used in the blot; both the pellet and the supernatant of each sample of MRSA and MSSA in the presence of increasing amounts of methicillin were blotted. However, there was no detectable protein of the correct molecular weight.

Figure 3.10 Western blot showing MRSA cells grown in two concentrations of methicillin and penicillin. The MIC of methicillin with this strain is 32 μg/ml, so it would be expected that PBP2a would be induced in these samples. These were not spun before labelling to investigate whether PBP2a was being lost at this point as there had been no detection of the protein. The samples on the gel are as follows: Lane 2, MRSA cells grown in the presence of 32 μg/ml penicillin; Lane 3, MRSA grown in 100 μg/ml penicillin; Lane 4, MRSA grown in 32 μg/ml methicillin; Lane 5, MRSA grown in 100 μg/ml methicillin; Lane 6, uninduced MRSA cells grown in the absence of antibiotics; Lane 7, MSSA cells grown in the absence of antibiotic; Lane 8, Bovine Serum Albumin standard (5 μg/ml). The Molecular weight standard (Bio-Rad) had been run in Lane 1 and was removed from the blot and stained with Amido Black so the bands could easily be visualised.

Note that a non-specific band is the only band detected.

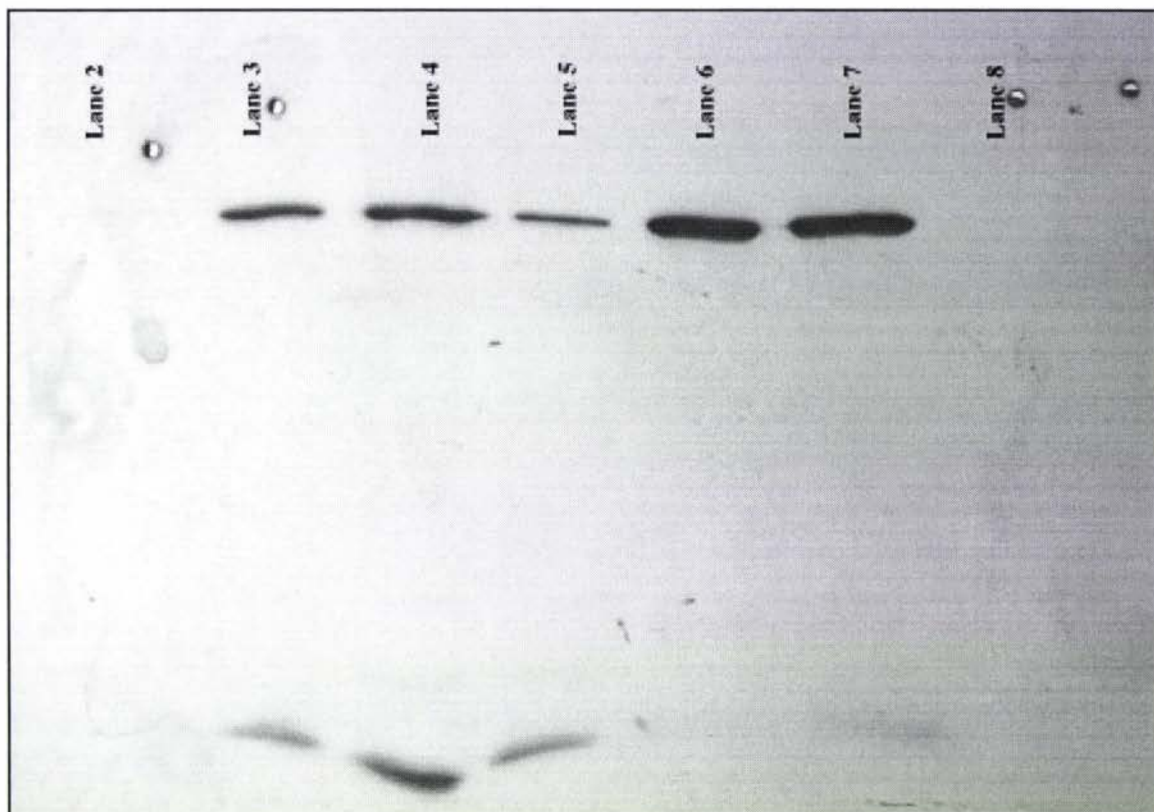
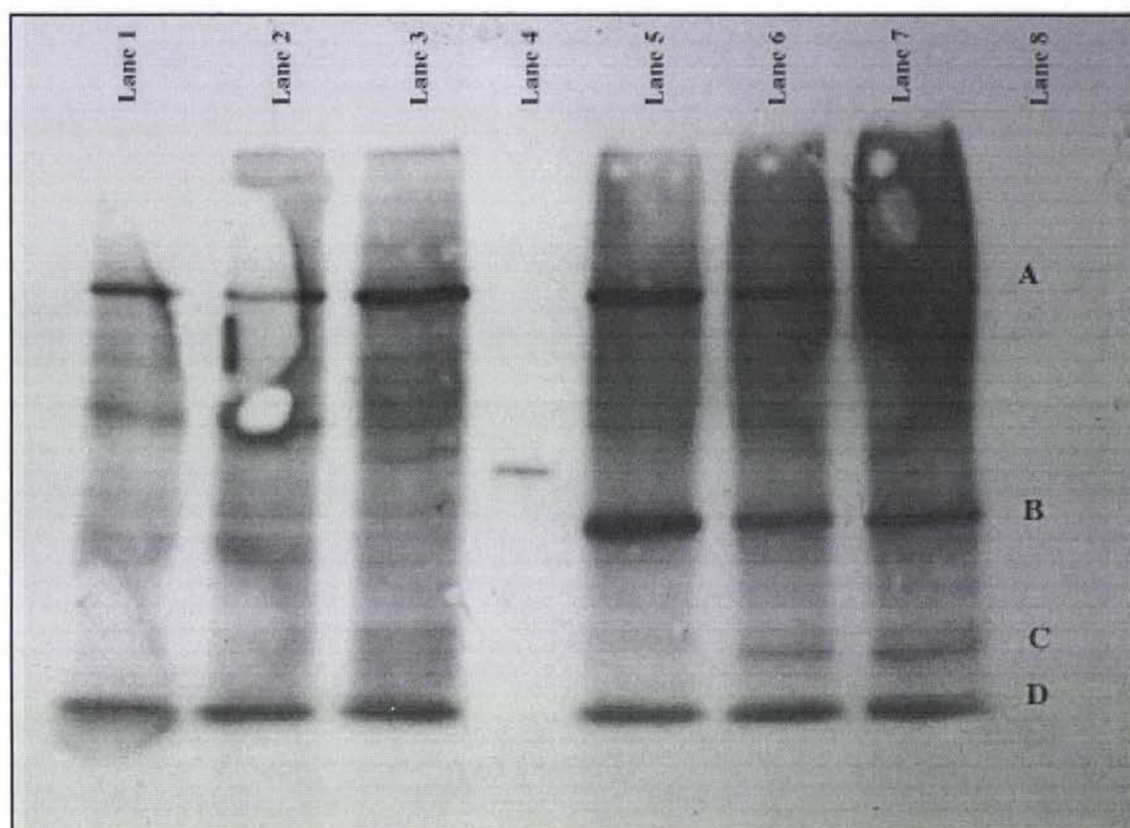

Lane 2
Lane 3
Lane 4
Lane 5
Lane 6
Lane 7
Lane 8

Figure 3.11 Western Blot showing cells labelled with 6-aminopenicillanic acid. Both MRSA and MSSA were grown in the presence of antibiotic in an effort to induce the production of PBP2a. Lanes are as follows: Lane 1, MSSA grown in 100 μg/ml methicillin; Lane 2, MSSA grown in 32 μg/ml methicillin; Lane 3, MSSA grown in the absence of antibiotic; Lane 4, Molecular weight standard (Bio-Rad); Lane 5, MRSA grown in the absence of antibiotic; Lane 6, MRSA grown in 32 μg/ml methicillin; Lane 7, MRSA grown in 100 μg/ml methicillin; Lane 8, Bovine Serum Albumin (5 μg/ml).

Note the appearance of a band present only in MRSA samples (B). However, it is too small to be attributed to PBP2a (78 kDa) as it is only around 50 kDa in size. There also appears to be a band that occurs in MRSA samples at higher intensities with higher concentrations of antibiotic, as would be expected for PBP2a (C). However, it is even smaller than the band discussed above. The same non-specific binding seen in Figure 3.10 is also present (A) and as well as another non-specific band (D).


Lane 1
Lane 2
Lane 3
Lane 4
Lane 5
Lane 6
Lane 7
Lane 8
A
B
C
D

At this point an attempt was made to obtain some anti-PBP 2a antibody so as to utilise another method by which to detect the protein. As it had not proved to be possible even to detect PBP 2a under induction conditions it was considered to be very unlikely it would be possible to visualise its disappearance in the presence of totarol so the addition of the diterpene to samples was not investigated further. The experiment was delayed until the arrival of the anti-PBP 2a antibody. As this did not appear before the end of this research project, the experiment was not attempted any further.

4. DISCUSSION

4.1 Determination of Inhibitory Activity of Diterpene Derivatives

4.1.1 Determination of MICs For Diterpene Derivatives with *S. aureus*

The MIC for each diterpene derivative was determined both for MRSA strain MR96/164 and MSSA strain 87. The derivatives with the lowest MICs for both strains were found to be totarol, 258, 390 and 416, having MICs ranging from 2 to 16 μg/ml (Table 3.1 and 3.2). All other diterpene derivatives had MICs of 32 or above, making them unsuitable as potential chemotherapeutic agents. Totarol had the lowest MIC for both strains at 2.0 for MRSA and 3.2 for MSSA. This was encouraging for future research. A low concentration such as 2 μg/ml would be easily achievable therapeutically; it has been found that totarol can be administered subcutaneously at such concentrations (Evans *et al.*, 1998) and a low MIC for other diterpene derivatives is also encouraging for potentiation activity as only a low concentration would be required to achieve potentiation of β-lactams.

The effect of structural groups on the activity of totarol derivatives was an important aspect of this research. Although totarol possessed the greatest independent activity, the three derivatives with the lowest MICs, Derivatives 258, 390 and 416 were relatively similar in structure, all being differing from totarol by the addition of a single functional group. Derivative 258 was synthesised by the addition of a methyl alcohol group to totarol; Derivative 390 was made by the addition of a hydroxyl group and Derivative 416 differed from totarol by virtue of the addition of a methyl group to the parent compound. Derivatives 258 and 390 would both be expected to be slightly less lipophilic with the addition of hydrophilic side chains. However, this did not result in increased activity of either of these derivatives against either of the Gram negative bacteria tested.

The lipophilicity of derivatives may also be important for their potential use as chemotherapeutics. In a trial conducted by Evans *et al.* (1998) on mice injected subcutaneously with totarol it was found that although the diterpene exerted no toxic effects on the animals and protected against a potentially lethal dose of MSSA, the diterpene was very poorly bioavailable, making it difficult to evenly distribute it throughout the body. It was suggested that increased bioavailability such as that of derivatives including totarol α-D-mannopyranoside or totarol β-lactoside, generated in that research, might result in improved activity. However, this was not observed. Although the un-ionised form of totarol is very lipophilic and can be improved by glycosylation, the activity of such derivatives was reduced. As a result it would seem important to produce derivatives of totarol with side chains to reduce the lipophilicity of the compound, retaining the antibacterial activity of the diterpene, such as the compounds described above.

4.1.2 Disk Diffusion Tests for Diterpene Derivatives with *H. pylori*

Due to the difficulties inherent in the liquid culturing of *H. pylori*, disk diffusion tests were employed to investigate the activity of diterpene derivatives against this bacterium. In this method the activity of each derivative was compared by measuring the lowest amount of each derivative required to cause a zone of inhibition around the antibiotic disk. Only 3 derivatives, 289, PK2 and PK16, caused a zone of inhibition when the lowest amount of compound, 1 μg, was present and these ranged from 6.5 to 8 mm in diameter. The diameter of the disk itself was 6 mm, so the resulting zone of inhibition was very small, representing little antibacterial activity.

Xia *et al.* (1994) published guidelines to standardise the classification of *H. pylori* as sensitive or resistant to metronidazole, an antibiotic commonly used to treat *H. pylori* infections, by this method. Xia *et al.* (1994) used antibiotic disks containing 5 μg metronidazole. It was reported that the zone of inhibition must be greater than 20 mm to indicate sensitivity to the antibiotic. Only one derivative, PK16, had a zone of inhibition greater than 20 mm at any concentration. A zone of inhibition of 22 mm

was observed for this derivative at the highest level tested, 50 μg. If the standardisations described by Xia *et al.* (1994) are adopted for this experiment, the zone of inhibition seen with derivative PK16 indicates only borderline sensitivity. This derivative possessed little or no activity against *H. pylori* as indicated by the finding that 50 μg of derivative was required to achieve a zone of inhibition greater than 20 mm. This is ten times the amount of metronidazole recommended by Xia *et al.* From these results it was determined that none of the derivatives possessed any significant activity against *H. pylori.*

The three derivatives exhibiting the most activity against *H. pylori*, 289, PK2 and PK16, were also tested with *E. coli* in the same method as for *S. aureus*, standard macrobroth dilution, to ensure that the lack of activity seen with *H. pylori* was not due to the different method used. However, none of the derivatives showed any activity against *E. coli*. This was partially expected as previous researchers have determined that totarol and other derivatives possess no significant activity against Gram negative bacteria (Bendall and Cambie, 1995; Kubo, *et al.*, 1992). The finding that pisiferic acid does inhibit the growth of Gram negative bacteria (Kobayashi *et al.*, 1988) distinguishes it from other diterpenes.

When the structural groups of these three derivatives are considered, it can be seen that Derivative 289, synthesised from podocarpic acid, a compound related to totarol, has the addition of an ethyl group to the hydroxyl group present in podocarpic acid. Derivative PK2 has a hydroxyl group and the carbon ring present in totarol has been cleaved. Derivative PK16 is similar in structure to Derivative PK2, with cleavage of the carbon ring present in the parent compound, and also has a hydroxyl group added. There is also a ketone group in this molecule. It was postulated that compounds with less lipophilic structures would be more effective against Gram negative bacteria as the Gram negative cell membrane is impermeable except through water-filled channels, making it impossible for lipophilic compounds to pass through the membrane. However, this relationship does not seem to be significant in this research and it was not possible to determine a structure/function relationship for these compounds against these bacteria. None of the diterpene derivatives tested

were significantly more active in a manner which could be related to a particular substitution of functional groups.

4.2 Potentiation Activity of Diterpene Derivatives

4.2.1 Potentiation of Methicillin Activity Against MRSA

The MIC for methicillin had previously been determined to be 32 μg/ml. In order to investigate whether diterpene derivatives could potentiate the activity of the β-lactam and reduce the MIC for MRSA, serial dilutions of methicillin were augmented with 10 μg/ml or half-MIC, whichever volume was less, of each diterpene derivative and inoculated with MRSA. Significant potentiation activity was observed in seven derivatives, reducing the MIC down to below 0.125 μg/ml in the case of derivative 416. The remainder of derivatives had MICs ranging from 16 to greater than 32 μg/ml.

The derivatives possessing the greatest potentiation activity and thus the most potential as adjuncts for β-lactams were totarol and PK15 (4 μg/ml), 390 (<2 μg/ml) and 416 (<0.125 μg/ml) These derivatives were to be investigated further to attempt to determine the mode of action by which this activity occurs. It was also observed that although these derivatives significantly reduced the MIC for methicillin, none were able to reduce the MBC which remained greater than 32 μg/ml for all diterpene derivatives tested. This could affect the potential use of these compounds as chemotherapeutic agents as the bacteria are only being inhibited, not killed, at these lower concentrations.

The structures of derivatives 258, 390 and 416 were discussed previously (Section 4.1.1). The other derivatives also found to possess significant potentiation activity, Derivatives PK12, PK14 and PK15 were similar in structure. All were derived from totarol and had their aromatic ring cleaved. Derivatives PK12 and PK15 have a hydroxyl and methyl alcohol group exposed as well as short hydrocarbon chains.

Derivative PK14, with two cleaved aromatic ring structures, has a hydroxyl group and short-length hydrocarbon chain attached to the same aromatic ring as Derivative PK12 and Derivative 15, and a methyl alcohol group added at the site of cleavage of the second broken ring. All these have the effect of reducing the hydrophobicity of the compounds and also indicate that the preservation of the aromatic ring structure is not necessary to maintain potentiation activity, although these three compounds had lower independent activity, with MICs of 32 μg/ml for the MRSA strain tested in this study.

4.2.2 Potentiation of methicillin activity against *E. coli*

Three derivatives were tested for potentiation activity against this bacterium. These three derivatives were selected as they possessed the most activity independently; the derivatives showing the best potentiation activity against MRSA also possessed the most independent activity. However, none of the derivatives reduced the MIC for methicillin against *E. coli*; it remained greater than 32 μg/ml for all derivatives tested. This further reinforced the observation that none of the derivatives tested possessed any activity against Gram negative bacteria. This was as expected as previous studies on diterpenes have indicated that with the exception of pisiferic acid, they do not possess activity against Gram negative bacteria (Bendall and Cambie, 1995; Kubo *et al.*, 1992; Kobayashi *et al.*, 1988). However, it was felt important to investigate the potentiation effect of derivatives on Gram negative bacteria as the rational derivatisation of diterpenes to produce compounds with increased activity against this group of bacteria was an important aspect of this research. As this did not prove to isolate derivatives possessing significant Gram negative activity, further research was conducted on *S. aureus* as the test Gram positive organism and investigation on Gram negative bacteria was abandoned.

4.3 The Effect of Totarol on DNA Synthesis

The rate of incorporation of tritiated methyl-thymidine, a precursor to DNA, was used as an indicator of the rate of DNA synthesis of MRSA strain MR96/164 in the presence of increasing concentrations of totarol. Three methods were used to measure the growth of the culture over this period in an investigation to determine a method giving a quantitative rate of thymidine incorporation.

It was found that there were significant problems in the accurate determination of the rate of thymidine incorporation when the first method, determination of viable cell count, was used. As Figure 3.1 showed, there was much higher incorporation per cell in the two highest concentrations of totarol. This trend appeared in all experiments carried out under these conditions, so was not simply an artefact of the data presented in the above figure. This high rate of incorporation by cells at these concentrations of totarol could be interpreted to mean that the DNA synthesis mechanisms are unaffected by totarol at MIC levels, and that the cells in these concentrations of totarol are synthesising the molecule at normal rates, until killed by the diterpene. As the ratios of incorporation per viable cell in the other, lower concentrations of totarol are lower, it could be inferred that this meant that although DNA synthesis was unaffected by totarol, another cellular target was in fact being inhibited by the diterpene, causing an overall decrease in the biosynthetic activity of the samples and hence lower rates of incorporation per cell due to this inhibition of another cellular target.

However, this method was felt to be inaccurate for the following reasons. At the highest concentrations of totarol, very few viable cells remain over the last two or three sample times. As a result, incorporation measured by this method was being estimated to be carried out only relative to these few cells. It did not take into account any incorporation by cells between samples, that were no longer viable when the culture was sampled. This caused the rate of incorporation per cell to appear much higher in these cultures. Although it was anticipated that the actual trend in incorporation by the culture would be similar, this method had the effect of

magnifying it. It was decided to use another method to measure the growth of the culture that could take into account any incorporation by cells that did not remain viable at sample times, that is measure the growth of the whole culture, viable and non-viable cells.

Measurement of the dry weight of the culture was the next method utilised to measure the growth of the culture. However, this did not prove suitable for the experiment either. Initially 0.1 ml samples were taken at each time point and immediately filtered onto nitro cellulose filters. The volume was increased to 0.5 ml when it was found that 0.1 ml did not yield sufficient mass to be measured. However, it was subsequently found that 0.5 ml samples also did not contain enough biomass to measure either. Although cultures were grown over a three hour period, they were incubated in stationary waterbath as no shaking waterbath was available, and were only shaken when sampled. This, coupled with the fact that totarol was added after 90 minutes with the specific aim of inhibiting growth, meant that the cultures only grew relatively slowly.

It may have been possible to use dry weight determination as an accurate measurement of the cultures' growth if larger sample volumes, for example 5 ml, had been taken. However, there were financial limitations on the amount of isotope that could be purchased and it was not possible to use cultures of the volume this sample size would require for the experiment.

The final and ultimately most appropriate method of determination of the biomass of the culture was a protein assay (Bio-Rad), measuring the total protein in the culture and therefore also the growth of the culture, providing a measurement of the total culture so as to avoid the bias seen in viable cell experiments. As described in Section 3.5.3, the first adaptation of this method, the microassay for microtitre plates, was not suitable as it measured a range of protein concentrations greater than that actually seen in the experiment. The standard microassay protocol was instead adopted, measuring protein concentrations ranging from 1 to 10 μg/ml protein.

Although this procedure was appropriate for the experiment, there were several difficulties met in refining the procedure. As the samples were incubated in LB (Section 2.2.3) it was necessary to use this in the preparation of standards as well. However, this proved to create a problem with background absorbance and it was necessary to boil LB for standard preparation for 10 minutes, as samples were, to prevent this.

The procedure was also problematic in the measurement of the absorbance of samples. In some instances there were abnormal readings for samples, with some absorbances falling either much above or below that of others. However, it was still possible to measure the total protein in the cultures accurately if these samples were disregarded and the results considered as a whole. Figure 3.3 presents typical data generated in an experiment using this method of culture growth measurement. The very high rate of incorporation seen in the 0.5 μg/ml totarol culture is an artefact of a very low absorbance measured for that sample in the protein assay. However, it is reasonably clear that the rate of incorporation of thymidine per microgram of protein throughout the cultures is at approximately the same level throughout the experiment. This result was observed in each experiment measuring the rate of incorporation by determination of the total protein of the culture. It would indicate that the mechanisms for DNA synthesis are functional in all the cultures at all the time points, suggesting totarol does not inhibit DNA synthesis as a primary mode of action. It would be anticipated that if inhibition of DNA synthesis were the main target of the diterpene, there would be significantly lower rates of incorporation in the cultures containing totarol at MIC levels. This was not seen in any of the experiments conducted on the investigation of DNA synthesis.

4.4 The Effect of Totarol on Protein Synthesis

The rate of incorporation of tritiated glutamine, a precursor for protein, was used to investigate the effect totarol exerted on protein synthesis in MRSA. As with the experiment on DNA synthesis, the first method employed to measure this was the

determination of the rate of incorporation per viable cell. Similar effects were seen in these experiments as those observed in the thymidine incorporation experiments. In each experiment, the rate of incorporation per cell was very high at the two highest concentrations of totarol because there were very few viable cells left and incorporation by all cells, including those that died before sampling, was being attributed to these few cells. However, the fact that cells exposed to the high concentrations of totarol were still incorporating glutamine indicated that the machinery for protein synthesis was still functioning and hence that inhibition of protein synthesis was not the primary mode of action of totarol.

Due to the inaccuracy described above, another method was employed to further investigate the effect of totarol on protein synthesis. The problems previously encountered in dry weight determination eliminated this method from use here and determination of total protein was instead used. Again, a similar result to that obtained in the thymidine incorporation experiments was seen. The rate of incorporation of glutamine per microgram of protein was similar in all concentrations of totarol tested. There was not significantly less incorporation in the presence of high concentrations of totarol as would be anticipated if inhibition of protein synthesis was occurring. However, the same problem with aberrant absorbances of samples in the protein assay was present in this experiment. As can be seen in Figure 3.5, there is one point in the control tube that is much higher than the other readings. This was due to an unusually low absorbance for that sample in the protein assay. However, if this single point is disregarded it is apparent that there is no significant inhibition of glutamine incorporation in the presence of increasing concentrations of totarol, and hence it can be inferred that inhibition of protein synthesis is not the primary target of totarol.

However there is a problem as protein levels are being used as reference points in an assay on protein synthesis. This experiment could be repeated using another marker, unrelated to protein synthesis, of cell growth to ensure there is no effect on the results seen due to the use of this marker.

4.5 The Effect of Totarol on Peptidoglycan Synthesis

The rate of incorporation of tritiated N-acetyl glucosamine was used to measure peptidoglycan synthesis in MRSA cells exposed to the same concentrations of totarol as those used for DNA and protein synthesis investigations. The preliminary experiments conducted to determine the most efficient way of recovering the labelled peptidoglycan and to ensure it was indeed peptidoglycan synthesis that was being measured, revealed that the most efficient method of recovery was to boil the cells in SDS to lyse them and then filter them directly onto glass filters. The rate of incorporation in control conditions was significantly higher than when the cells were grown in the presence of 1 μg/ml (half-MIC) vancomycin, indicating that incorporation of N-acetyl glucosamine into peptidoglycan was indeed being measured.

The results of these experiments were similar to those observed in the investigations of the effect of totarol on DNA and protein synthesis. The rate of incorporation of N-acetyl glucosamine per microgram of protein was similar in all concentrations of totarol tested. Again this would indicate that the machinery for peptidoglycan synthesis remains intact in the presence of MIC levels of totarol. This in turn indicates that the primary mode of action of the diterpene is not the inhibition of peptidoglycan synthesis.

These experiments were conducted using only the protein assay to measure the growth of the culture as this was the last incorporation experiment conducted and that method had previously been determined to be the most appropriate.

It has been suggested that totarol may act by inhibiting the expression and function of PBP 2a (Chamberland *et al.*, 1995) when in potentiation conditions. It was anticipated that this might be reflected in subsequent inhibition of peptidoglycan synthesis as PBP 2a is an important cell wall protein. However, no such inhibition was apparent in this investigation and the effect of totarol on PBP 2a specifically was investigated in a later section.

4.6 The Effect of Totarol on Cellular Respiration

The effect of totarol on the respiration of MRSA strain MR96/164 was investigated by measuring the rate of respiration, as microlitres of oxygen consumed per minute, in the presence of increasing concentrations of totarol. A range of concentrations of totarol from 7×10^{-5} mM (0.01 x MIC, 0.02 μg/ml) to 3.5×10^{-2} mM (5 x MIC, 10 μg/ml) was tested. It was discovered that totarol did in fact inhibit respiration at concentrations of 3.5×10^{-3} mM (1 μg/ml, 0.5 x MIC) and above, a concentration almost ten times smaller than the lowest determined to cause inhibition by Haraguchi *et al.* (1996).

The other study undertaken in this area, by Evans *et al.* (1998) measured the respiration of rat liver mitochondria in the presence of totarol, determining an uncoupling effect at low concentrations and an inhibitory effect at high concentrations of totarol. A result similar to that of Evans *et al.* (1998) was observed in this study, that at concentrations below 1 mM (0.286 μg/ml, 0.143 x MIC) there was an increase in respiration by MRSA. Initially it was thought that this was due to the uncoupling effect described in mitochondria by Evans *et al.* (1998) as it was occurring at similar low concentrations of the diterpene.

All totarol dilutions were made in methanol and stored at -20°C. It seemed possible that at low concentrations the apparent uncoupling activity may actually be due to the methanol; at the lowest concentrations of totarol, the solution was made almost entirely of methanol due to the dilution of totarol. This possibility was therefore investigated by conducting an experiment where only 17.5 μl of methanol was added to the culture in the chamber and its effect on the respiration of the bacterial suspension monitored. There was still an increase in respiration, of approximately the same magnitude as that observed in the low concentrations of totarol, in this experiment. This indicated that the increase in respiration in these concentrations of totarol were in fact an artefact due to the methanol, probably uncoupling. This is rationalised as permeabilisation of bacterial membranes to protons, partially

depleting the transmembrane potential and therefore requiring an increase in respiration to compensate.

This phenomenon also disappeared as the concentrations of totarol became stronger; at an intermediate concentration of totarol that was tested (1 x 10^{-3} mM, 0.143 x MIC, 0.286 μg/ml) there was neither inhibition nor a significant increase in respiration rate. It appeared that at this concentration the stimulatory or uncoupling effect of the methanol was being counteracted by the inhibitory effect of totarol. As the concentrations tested progressed above this concentration there was no increase in respiration and the rates of respiration measured decreased with the increasing concentrations of totarol.

Although there is a slight respiration measured for the highest concentration tested, 5 x MIC (10 μg/ml), there was in fact total inhibition of respiration. The cells respired a very slight amount for the first minute after the addition of totarol before respiration was completely inhibited for the remainder of the experiment, resulting in the very low rate of respiration that appears on the graph in Figure 3.9. There was however complete inhibition. Moreover, we are confident that respiration was being measured in these experiments as the addition of cyanide always resulted in a significant reduction of the rate of oxygen depletion from the chamber, indicating inhibition of the respiratory chain.

These results would then indicate that totarol does in fact inhibit respiration in MRSA. This can be proposed as a relevant mode of antibacterial activity as there is significant inhibition at MIC levels (around 70 % inhibition) and inhibition increases with increasing concentrations of totarol. The initial increase in respiration that was observed at low concentrations of totarol can be attributed to the effect of the methanol that the totarol was stored in as the same effect was observed when methanol only was added to the chamber.

Previous research conducted by Haraguchi *et al.* (1996) suggested that the primary mode of action of totarol was the inhibition of respiration after measuring the rate of

respiration of *S. aureus*, *P. aeruginosa* and *M. luteus* in the presence of totarol. The respiration of *E. coli* was found to be unaffected by totarol. However, this conflicted with the findings of Evans *et al.* (1998) who conducted experiments on the effect of totarol on respiration using rat liver mitochondria, and determined that totarol did inhibit respiration, but only at high concentrations, above MIC level. The concentrations investigated by Haraguchi *et al.* (1996) were above MIC level. Evans *et al.* (1998) found that at MIC levels totarol was not toxic for HeLa cells and that at these concentrations the only effect observed in mitochondria due to totarol was a slight uncoupling effect, resulting in slightly elevated oxygen consumption rates. It was also determined that at MIC levels totarol did not show any cytotoxic effects on human cells and a subcutaneous dose of totarol was protective against a potentially lethal dose of MSSA in a mouse model (Evans *et al.*, 1998). The aim of this part of the research was then to determine whether totarol did or did not have an effect on respiration.

The results obtained in this section seem to concur with those obtained by Haraguchi *et al.* (1996) and it can be concluded that the primary mode of action of totarol is inhibition of respiration. Inhibition was found to occur at lower concentrations than those tested by Evans *et al.* (1998). The substrate used by Evans *et al.* in the investigation of the effect of totarol on respiration were mitochondria from the livers of rats, so cannot be directly compared to those obtained by Haraguchi *et al.* (1996) and inhibition was found to occur at higher concentrations, above 5×10^{-2} mM. Totarol at this concentration completely inhibited the respiration of MRSA cells. The apparent resistance exhibited by mitochondria may be due to the fact they are eukaryotic organelles and as such differ in the structure of their membranes, which may result in the higher level of resistance exhibited.

The different concentrations required for totarol to inhibit respiration in the different cells may be advantageous for its potential use as a chemotherapeutic. Evans *et al.* (1998) report that human cell lines are unaffected by totarol to concentrations of 3×10^{-2} mM. The respiration of MRSA cells is inhibited by totarol at concentrations as low as 3.5×10^{-3} mM (1 μg/ml, 0.5 x MIC). This would suggest that the bacteria

could easily be inhibited at concentrations well below those that are toxic to human cells. This is particularly encouraging for the potential use of totarol as an adjunct to β-lactam antibiotics, to potentiate their activity. Totarol has been found to significantly potentiate the activity of methicillin when added to a final concentration of 3.5×10^{-3} (1 μg/ml, 0.5 x MIC). From these results that level of totarol would potentially have no effect on human cells.

4.7 Investigation of the Effect of Totarol on PBP 2a Activity

A non-radioactive detection method (Dargis and Malouin, 1994) was used in an attempt to investigate the effect of totarol on the production and activity of PBP 2a in MRSA. This was selected as it would provide an alternative to the traditional method of isolating PBP 2a using radiolabelled penicillin and visualising the protein in X-ray films.

The general method used detects the protein by labelling a β-lactam antibiotic by binding it to an ester of biotin This labelled β-lactam binds to PBP 2a and the subsequently biotinylated protein is in turn detected with a streptavidin-horse radish peroxidase conjugate which binds to the biotin. The conjugate undergoes a light-emitting reaction when the reagents from the ECL DNA detection kit are added, enabling the visualisation of the protein on X-ray film. In this research this method was not found to work.

During the course of this research it did not prove possible to detect the protein in any samples using this method. There was a problem with non-specific binding in all samples, resulting in a band of strong intensity. However this corresponded to a non-specific band that was also seen in samples processed by Dargis and Malouin (1994).

There were no other proteins detected by this method that corresponded to the position where PBP 2a would be expected to occur. PBP 2a is present in all strains of MRSA and therefore should have been induced under the appropriate conditions.

It was not determined if the failure to visualise PBP 2a was because the protein was not being induced fully, or if the procedure was not detecting the presence of the protein when induced. However samples of MRSA bacteria were grown in concentrations of methicillin and penicillin at MIC levels and higher, which would be expected to induce production of the protein sufficiently. It therefore appears likely that the lack of results were due to a failure to detect the protein.

Many strategies were employed in an attempt to label and detect the protein including the use of more than one antibiotic at increasing concentrations in attempts to induce the protein fully; both methicillin and penicillin were used at concentrations up to 100 μg/ml. Two antibiotics were used to detect the protein in blots; both ampicillin and 6-amino penicillanic acid were conjugated to biotin to ensure that it was not the antibiotic that was at fault. Samples were also manipulated in as many ways as possible including growing cells to a higher density before the addition of antibiotic and the addition of antibiotic to cells at a lower density. Samples were also not centrifuged before being loaded on protein gels to investigate whether PBP 2a was being lost at this stage of the experiment. Samples were also labelled for 60 minutes instead of 30, as recommended by Dargis and Malouin (1994). There was still no positive identification of PBP 2a.

Although an attempt was made to obtain anti-PBP 2a antibody, it did not arrive until after the conclusion of this research. It was anticipated that using a specific detection method such as an antibody may have been more successful in the detection of the protein, thus facilitating an experiment to test the effect of diterpenes on its expression.

4.8 General Conclusions and Future Work

The mode of action of totarol was determined to be inhibition of cellular respiration, based on significant inhibition at half-MIC levels and above. Respiration was completely inhibited at 3.5×10^{-2} mM (10 μg/ml, 5 x MIC). This was supported by

the negative results of primary inhibition obtained in the experiments investigating the effect of totarol on DNA, protein and peptidoglycan synthesis. However, Haraguchi *et al.* (1996) determined that totarol does inhibit the synthesis of DNA, RNA and protein, as measured by incorporation of labelled precursors, to similar extents, which suggested interference with energy metabolism in a similar way to several respiratory-inhibiting antibiotics. The resulting inhibition of incorporation of precursors was observed because energy is required for the active uptake of various metabolites and for the biosynthesis of macromolecules. However, this was not seen in this research.

The finding that totarol inhibits cellular respiration agrees with the results of Haraguchi *et al.* (1996) who also determined this to be the mode of action. However, in this study it was found that inhibition occurred at lower concentrations than those used by Haraguchi *et al.* (1996). The discrepancy between the findings of this research and that of Evans *et al.* (1998) who determined that totarol did not inhibit respiration significantly at MIC levels is clearly due to the different cell types tested; Evans *et al.* (1998) tested the effect of totarol on cellular respiration using rat liver mitochondria. The different levels of totarol required to inhibit the different substrates are also demonstrated in the findings of the same researchers who determined that the growth of human cells was unaffected at concentrations that killed bacterial cells and that human cells could grow in much higher concentrations of totarol.

Some information was gained about the effect of structural groups on the activity of derivatives. It was observed that the derivatives determined to possess the greatest independent activity against MRSA had only small additions to their structure, remaining quite similar to the structure of totarol. Reduction of Lipophilicity was not found to significantly improve the activity of compounds against Gram negative bacteria, although the three derivatives exhibiting the greatest activity against *H. pylori* were found to possess some hydrophilic functional groups. It was also observed that the retention of the aromatic ring seemed to be important for

independent activity against MRSA, although significant potentiation activity was seen in three totarol derivatives with one or two rings broken open.

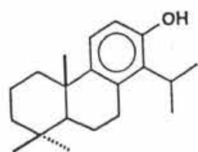
The evidence presented in this research would suggest that totarol is not harmful for human cells at MIC levels, which could make it possible for totarol to be used as a chemotherapeutic agent or an adjunct for existing β-lactam antibiotics. However, further research should be conducted on the effects of totarol on human cells to rule out toxicity at therapeutic levels. It should also be determined the exact way in which totarol inhibits respiration, determining which part of the respiratory chain is affected.

It would also be interesting to determine the effect of totarol and the other derivatives that demonstrated significant activity in this capacity, on respiration under potentiation conditions. This could easily be achieved by modifying the method used in this research to measure inhibition of respiration due to totarol. Methicillin could be added to the chamber and respiration measured before the addition of totarol or derivatives, added to a final concentration of half-MIC and respiration again measured to determine if there is inhibition. This would aid in the determination of the mode of action of the potentiation activity. The investigation of the efficacy of β-lactam antibiotics potentiated by totarol or derivatives is also important. It was discovered that the MBC of methicillin was not reduced by the derivatives in potentiation conditions; it should be investigated whether the *in vivo* activity of β-lactams will be sufficiently improved by the addition of diterpenes to eliminate an infection of MRSA.

It was not possible to determine the effect totarol has on the expression of PBP 2a in potentiation conditions in this research as the protocol could not successfully detect the presence of PBP 2a. However, with the arrival of anti-PBP 2a antibody, a Western Blot could be performed that is much more specific and any effect determined.

APPENDIX

A. Derivatives of Totarol



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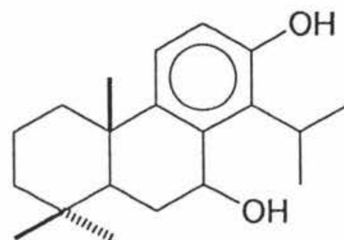

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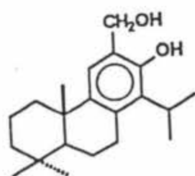

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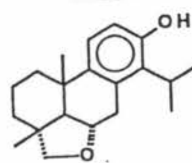

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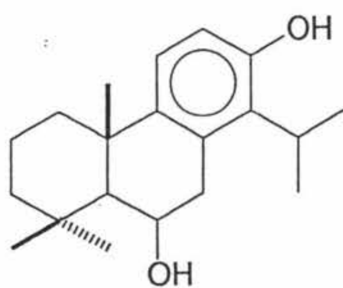

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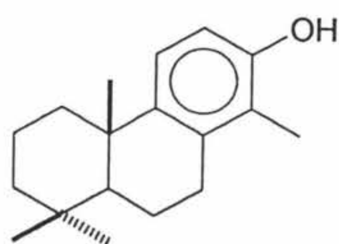

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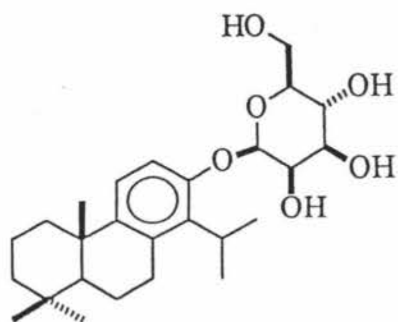

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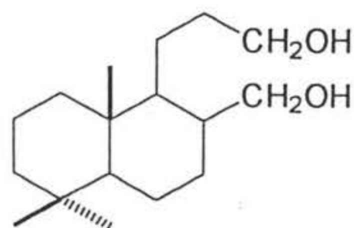

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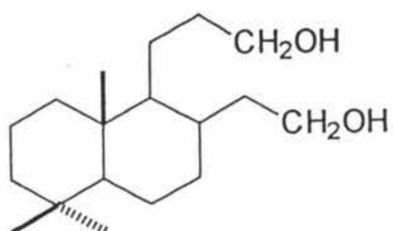

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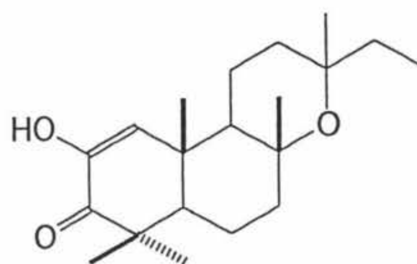

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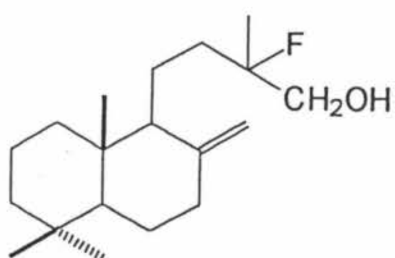

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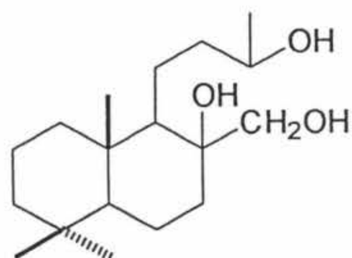

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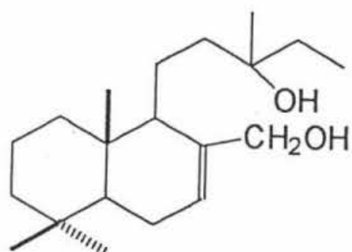

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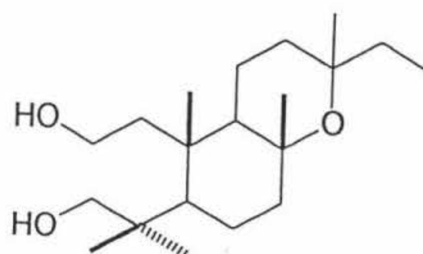

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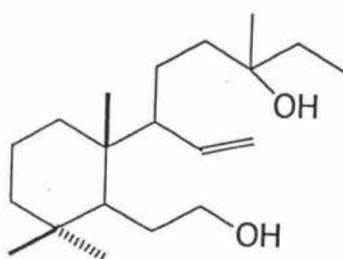

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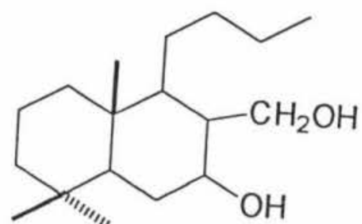

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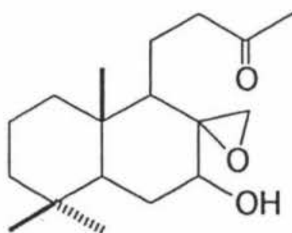

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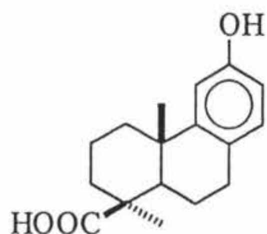
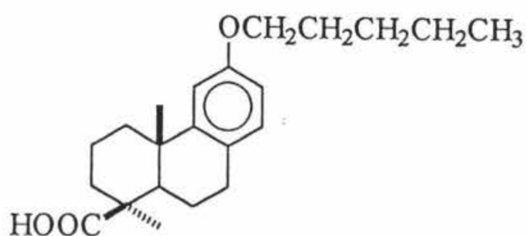
B. Derivatives of Podocarpic Acid



Podocarpic acid



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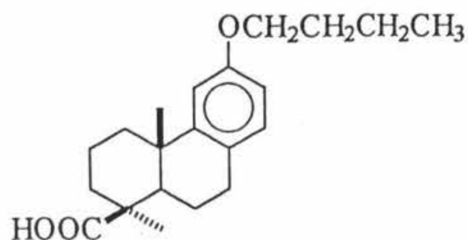

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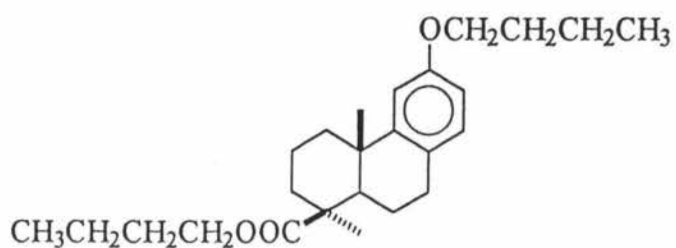

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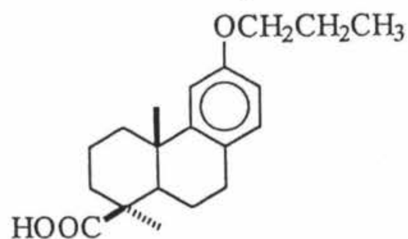

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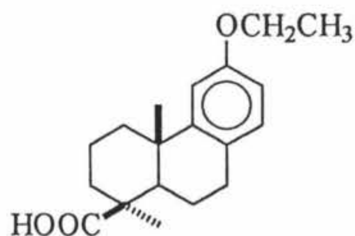

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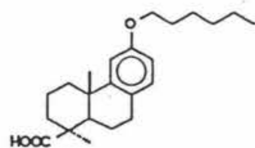

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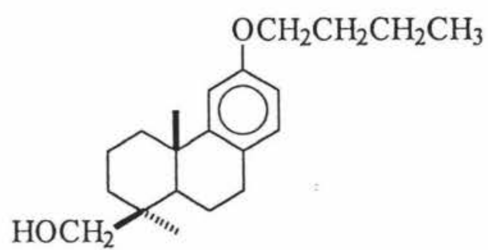

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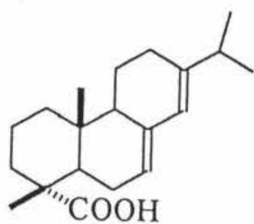
C. Diterpenes Extracted From Species of Pine



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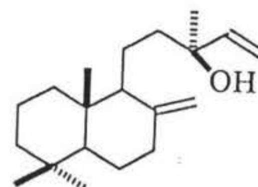

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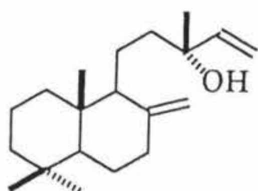

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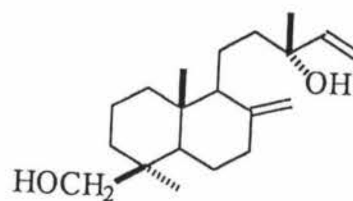

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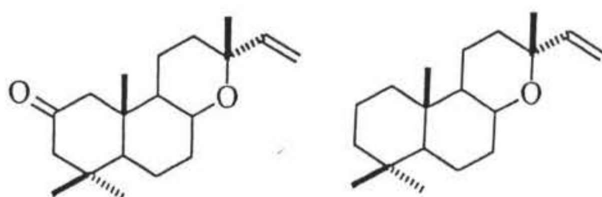

PK5

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