Production of bio-active compounds from ferulic acid using biocatalysis

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Since before time began
no one has ever imagined,
No ear heard, no eye seen, a God like you
who works for those who wait for him.

Isaiah 64:4

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Abstract

The use of ferulic acid, a ubiquitous plant phenolic compound for the production of compounds with biological activity was examined. This dibasic acid is an antioxidant and potential chemopreventative compound. The use of biocatalysis for the production of value added products such as vanillin and vanillic acid was investigated. The production of these flavour compounds from ferulic acid has been reported previously, using biocatalysis. The use of laccase isolated from *Trametes pubescens* and *versicolor* in organic media, for the production of vanillin and vanillic acid was unsuccessful. Another flavour compound, 4-vinylguaiacol was produced when the reaction was catalysed in ethyl acetate. Dimers of 4-vinylguaiacol were also observed. The expected polymerisation of ferulic acid by laccase in aqueous conditions was observed with the production of a previously undescribed ferulic acid tetramer, 3-carboxy-2'-(3-carboxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-5-yl)-5-(2-carboxyvinyl)-7,7'-dimethoxy-2,2',3,3'-tetrahydro-2,5'-bibenzofuran-3'-carboxylate.

Lipase-catalysed transesterifications of steroids were investigated using four compounds, β-estradiol, dihydrocholesterol, 5α-androstane-3β, 17β-diol, or arbutin, using lipases. Lipase B from *Candida antarctica* (Novozyme 435®), *Candida rugosa, Chromobacterium viscosum* and *Pseudomonas* sp. were used to perform the transesterification of chemically synthesized vinyl ferulate and trifluoro ethyl cinnamate. All the products were purified and characterised for antioxidant activity using three standard assays. Using the LDL assay, the arbutin ferulate product exhibited the highest activity and against the DPPH$^{\bullet}$ free radical, dihydrocholesterol ferulate. Using the TEAC assay the highest activity against the ABTS free radical was by 3β–O-feruloyl-17β-hydroxy-5α-androstane(**35**) (TEAC of 2.16).All the biocatalytically synthesised products exhibited higher antioxidant activity than Trolox, a well known commercial antioxidant and their precursor ferulic acid. Antioxidant synergy between ferulic acid and 3β–O-feruloyl-17β-hydroxy-5α-androstane was observed using the DPPH assay but none noted

in the TEAC and LDL assays. A new assay, the anti- NADH oxidase (NOX) assay was developed in order to asses the ability of the synthesised compounds to inhibit the redox protein NADH oxidase. This protein has been found to be tumour associated and is affected by the levels of cellular oxidation. The products from the laccase modifications of ferulic acid were found to have anti-NOX activity.

This study demonstrates the effective and successful use of biocatalysis for production of natural derivatives of ferulic acid with higher biological activity than the precursor. Naturally produced compounds are a much sought after alternative to synthetic chemical products. These compounds potentially have applications as drugs or drug precursors, and as result of their natural synthesis they have high economic and pharmaceutical value.

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1 Literature review

1.1 Introduction

Biotransformation is the process in which a compound is transformed into another using a biological system. While biotransformation may refer to the use of whole cells, biocatalysis is generally considered to be the conversion of substances by enzymes. The scope of biocatalysis is vast, ranging from enzyme-mediated synthesis of natural products such as polysaccharides or proteins, to the synthesis of fine chemicals from simple chemical feedstocks. Biotransformations are an effective and often preferable alternative to chemical synthesis for the synthesis of chemicals such as flavour compounds, pharmaceuticals, industrial feedstocks and optically active compounds.

Man has applied bioconversions for centuries. The brewing process, which dates back to 6 000 BC, (Smith, 1996) is the production of alcoholic beverages using, in most cases, yeast as the biological agent. Cheese making, is an archetype of biotransformation and biocatalysis as enzymes and microbes are used to mature milk proteins into the desired forms with characteristic taste and smell.

The unique properties of enzymes, such as their high chemo-, regio-, and enantioselectivity are highly advantageous in the synthesis of natural, agrochemical and pharmaceutical products. Their ability to work under mild reaction conditions, such as room temperature and neutral or almost neutral pH, greatly reduces costs that may be incurred, for example, in energy-consuming chemical processes. Enzymes can also be very efficient catalysts, capable of increasing reaction rates 10^{12} times (Carrea and Riva, 2000) and they are less hazardous to work with than conventional transition metal catalysts. This thesis will demonstrate the application of biocatalysis to produce value-added compounds from ferulic acid.

1.2 The nature of biocatalysts

Living organisms carry out thousands of chemical processes. In cells, protein biomolecules (enzymes and ribozymes), have the ability to catalyse the conversion of specific substrates to specific products in normal cell metabolic processes (Walsh, 2001). The most advantageous properties of enzymes arise from their remarkable specificities. The underlying basis for their chemoselectivity and regio- and stereospecificities is their molecular structural nature, which is predetermined by their protein structure, encompassing their primary, secondary, tertiary and quaternary structure (Garrett and Grisham, 1999). Enzymes only bind specific substrates, which explain their chemoselectivity. The chirality of their amino acid components provides an asymmetric microenvironment for substrate binding and transformation in the active sites (Walsh, 2001).

1.3 Biocatalysis for the production of fine chemicals

There is a wide diversity of enzymes and as a result, there are enzyme-catalysed equivalents of almost all types of organic reactions (Faber, 1995; Koeller and Wong, 2001 see Table 1-1). Even the Diels-Alder reaction, which was previously reported as being one of the exceptions, has been found to be catalysed by abzymes (catalytic antibodies) and ribozymes (Braisted and Schultz, 1990). Industries, for which the requirement for enantiomerically-pure compounds is crucial such as pharma, have benefited widely from this diversity of enzyme reactions, as their need for different catalysts has grown.

Table 1-1 Enzymes commonly used in organic synthesis (taken from Koeller and Wong, 2001)

Enzymes	Reactions
Esterases, lipase	Ester hydrolysis, formation
Amidases (proteases, acylases)	Amide hydrolysis, formation
Dehydrogenases	Oxidoreduction of alcohols and ketones
Oxygenases (mono and dioxygenases)	Oxidation
Peroxidases	Oxidation, epoxidation,halohydration
Kinases	Phosphorylation (ATP dependant)
Aldolases, transketolases	Aldol reaction (C-C bond)
Glycosidases, glycosyltransferases	Glycosidic bond formation
Phosphorylases, phosphotases,	Formation and hydrolysis of phosphate
Sulfotransferases	Formation of sulphate esters
Transaminases	Amino acid synthesis (C-N bond)
Hydrolases	Hydrolysis
Isomerases, lysases, hydratases	Isomerisation, addition, elimination, replacement

Enantiospecificity is very important in the pharmaceutical industry where biologically active compounds are usually chiral, and their enantiomers may not be active, or possess different activity as evidenced by the disaster caused by thalidomide. The R isomer of thalidomide is an effective sedative whereas the S isomer is tetragenic, leading to birth defects in the children of women who took the drug whilst pregnant (http://www.chm.bris.ac.uk/motm/thalidomide/optical2iso.html). In the flavour industry, enantiopure compounds are also required, as different enantiomers can have different sensorial properties (Brenna *et al.*, 2003).

Many of the substrates and products relevant in organic synthesis are hydrophobic in nature. Water is a problematic solvent in preparative organic chemistry because it has a high heat of vaporisation and boiling point, which makes recovery of products difficult (Faber, 1995). Furthermore, hydrolysis, racemisation, polymerisation and decomposition

are all favoured in aqueous media, but are often unwanted side reactions. In order to solve this problem, organic chemists preferentially use organic solvents (Faber, 1995). Traditionally it was thought that organic solvents destroy enzyme activity (Nelson and Cox, 2000). This has convincingly been shown not to be the case, as the application of biocatalysis in organic solvents has become widespread, and indeed, the usefulness of enzymes has been enhanced by applying them in organic media (Klibanov, 2001).

1.3.1 Biocatalysis in organic solvent systems

Both water-miscible and non-miscible organic solvents can be used for biocatalytic processes. Water-miscible organic solvents, such as dimethyl sulfoxide, tetrahydrofuran and acetone, can be used as co-solvents with water. These are often used in the biocatalysis of lipophilic compounds which are not soluble in an aqueous system alone and have a very low reaction rate (Faber, 1995). Enzymes are not very tolerant to water-miscible solvents because they lose catalytic activity. Hence, these water-miscible cosolvents have to be used at low concentrations (generally up to 10%) and thus their applications are limited.

There are three main types of applications of organic enzyme systems using water-immiscible solvents in biocatalysis (Faber, 1995):

1.) Biphasic systems: Hydrophilic constituents of the reaction, as well as the enzyme, are contained in the aqueous phase and hydrophobic constituents remain in the organic phase;
2.) Inverse micellar systems : The system is composed of homogenous thermodynamically stable, reverse micelles containing hydrated enzyme molecules in organic solvent; and
3.) Organic solvent systems (also known as non-aqueous enzymology): Immobilised enzyme preparations are freely suspended in organic solvent. A small amount of water (less than 5% v/v) is present in order to allow enzyme mobility and hence to maintain enzyme activity.

These systems are illustrated diagrammatically below in Figure 1-1.

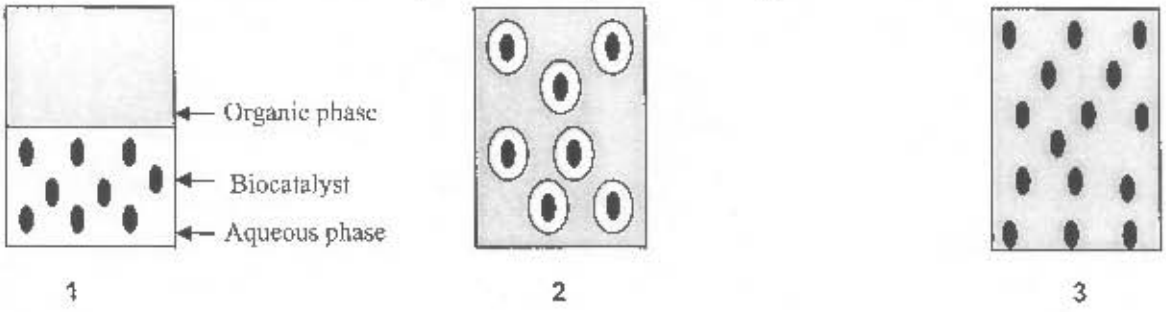


Figure 1-1. Representation of organic water-immiscible solvent systems. White areas represent aqueous phase, black dots immobilised enzyme and grey represents organic solvent. Biphasic system (1), inverse micellar system (2), and organic solvent system (3) (Taken from Carrea and Riva, 2000).

1.3.1.1 Biphasic biocatalytic reaction systems

Biphasic reaction systems are favoured when there is a difference in hydrophobicity between substrates and their products. The presence of two phases can increase the turnover of the reaction as the removal of the product from the enzyme surface can drive the reaction to completion (Faber, 1995). Enzymes are prone to substrate or product inhibition, and a biphasic reaction system may alleviate this problem by allowing higher concentrations of substrates to be used as the substrates or products separate into a different phase, lowering their concentration in the environment of the enzyme. A classic example is that of the decarboxylation of ferulic acid by whole cells of *Bacillus pumulis* (Lee *et al.* 1998). In a biphasic reaction system ferulic acid decarboxylase catalyses the decarboxylation of ferulic acid, but is subject to both substrate and product inhibition. The substrate, ferulic acid, is hydrophilic, whereas the product, 4-vinylguaiacol, is lipophilic. The product yield was reported to be 10-fold higher in the biphasic reaction system of buffer and hexane (Lee *et al.*, 1998).

Efficient mass transfer is necessary in biphasic reaction systems (Lilly, 1982). The distribution of the substrates and products could potentially present a rate-limiting factor and thus the partition co-efficient and the mass transfer coefficient could dominate over

the k_{cat} of the enzyme in influencing yield. Thus, the overall reaction rate would be dependent on the physical properties of the system rather than the enzyme catalytic power (Faber, 1995). This may result in a limitation on the reaction rate if the mass transfer is insufficient. Thus, stirring is essential in order to improve the mass transfer rate. However, inactivation of the enzyme can result if the stirring rate is too high. Biphasic reaction systems can successfully transform hydrophobic substrates such as fats and steroids (Faber, 1995).

1.3.2 Non aqueous enzymology - Monophasic biocatalytic reaction systems

Enzymatic reactions conducted in organic solvent with up to 98 % bulk water removed are termed monophasic reaction systems and the method is referred to as non-aqueous enzymology (Zaks and Klibanov, 1988). It is interesting to note that the first reports of enzyme reactions in organic media were published in 1900 (Kastel and Loevenhart, 1900). However, research in this field only developed about 80 years later (Klibanov, 1986). Proteins are unable to unfold in non-aqueous media due to the low dielectric of most organic solvent.. Coupled with an increase in protein intramolecular hydrogen bonding, this effect results in enzymes being trapped in their native conformation, allowing them to remain catalytically active (Hartsough and Mertz, 1993). As a result, protein stability in organic solvents is different from that in water. Substrate-enzyme interactions are dependent on water, and thus it follows that the substrate specificity of substrates in organic solvents would be different from that in water (Zaks, 1996). This phenomenon presents the possibility of many interesting applications and is explored further in the following section (Section 1.3.2.1) on medium engineering.

Several advantages exist for conducting enzyme reactions in monophasic organic solvents, some of which will be explored further in the proceeding sections, including:

- Increased thermostability: at low water content, the thermostability of the enzyme is often increased in (Carrea and Riva, 2000; Zaks, 1996);

- Recycle and recovery of the insoluble enzyme: the enzyme can be easily recovered and possibly reused in some cases, especially if immobilised;
- pH: pH is an important factor in aqueous enzymatic reactions. In organic solvents a phenomenon known as 'pH memory' has been found to govern enzyme catalytic processes, in which enzymes behave in a manner that reflects the pH they were last exposed (Zaks and Klibanov, 1988; Klibanov 2001); and
- Reversal and control of enzyme activity: This is explained in the following section (section 1.3.2.1).

1.3.2.1 Medium engineering

A very significant advantage of conducting biocatalysis in organic solvent is the potential to shift thermodynamic equilibria, to reverse 'natural' enzyme reactions that are unfavourable in water. This allows, for example, synthesis reactions to take place instead of hydrolysis by lipases, esterases and proteases (Faber, 1995). These reactions are very slow in aqueous systems, but in anhydrous solvents, with the addition of alternative neutrophiles such as alcohols, amines, and thiols, transesterification, aminolysis and thiotransesterifications, respectively can take place (Zaks and Kilibanov, 1985). In addition, it is possible to manipulate enzyme selectivity using different solvents, which can provide an alternative to protein engineering (Wescott and Klibanov, 1994). An example of solvent-dependent enantioselectivity has been demonstrated by lipase-catalysed desymmetrization of prochiral dihydropyridine dicarboxylates, shown in Figure 1-2. The S-monoesters were obtained when di-isopropyl ether was used and the R-isomer formed when cyclohexane was used (Hirose *et al.*, 1992). Various hypotheses have been formulated to account for the solvent effects on enzymes and the mechanisms are thought to be due to more than a single mechanism (Carrea and Riva, 2000).

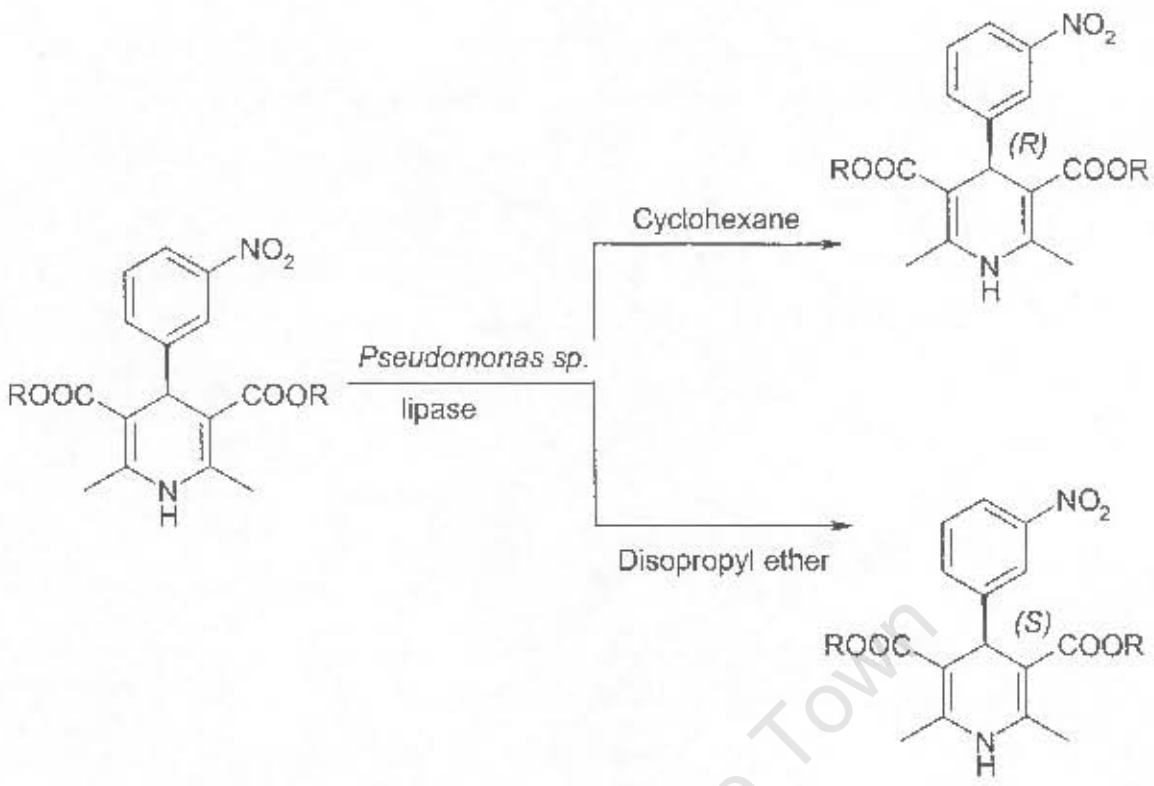


Figure 1-2. Lipase catalysed asymmetric hydrolysis of dihydropyridine carboxylates using medium engineering (Hirose *et al.*, 1992), adapted from Carrea and Riva (2000).

It is clear that the use of enzymes as biocatalysts has been accepted due to the range of applications and advantages that are possible. Many industrial processes have been adapted to use solely, or in part, biocatalysis, for example, in the fields of amino acid production and polymer synthesis (Klibanov, 2001; Koeller and Wong, 2001; Walsh, 2001). The use of enzymes as biocatalysts also provides insight that can be invaluable to synthetic organic and inorganic chemists attempting to reproduce and expand nature's chemical processes (Walsh, 2001).

This thesis will explore the use of biocatalysis for the production of bioactive compounds, specifically antioxidants, from ferulic acid, a plant phenolic compound.

1.4 Free radicals and antioxidants

Free radicals and other reactive oxygen species (ROS), are the causative agents in pathogenesis of various diseases (Halliwell *et al.,* 1995) and normal physiological phenomena such as aging (Lachance *et al.,* 2001). The aim of the work covered in this thesis was to develop antioxidant compounds that are effective against free radicals and their negative physiological effects.

Some diseases, such as cancer and the age related Alzheimer's disease, have been shown to be caused by free-radical mediated damage. Free radicals from various sources have damaging effects on cellular substructures and components such as membranes. The damage caused by free radicals, ultimately, results in disease (Figure 1-3). ROS, such as H_2O_2 and oxygen radicals, including the superoxide anion, $O^{\cdot}_2^{-}$, and peroxynitrite $ONOO^{-}$, are highly reactive chemical species that arise as the products of oxidative metabolism or life style. Oxidation also affects food, and this is one of the major sources of chemical spoilage (Jos and Huis, 1996). Almost half of worldwide fruit and vegetable spoilage is due to post-harvest degenerative oxidation reactions (Martinez and Whitaker, 1995). The oxidation of fats and oils in food results in rancid odours and flavours, leading to a deterioration of the nutritional quality, colour, texture and flavour of the foodstuff (Shahidi *et al.,* 1992), and further, secondary, potentially toxic compounds are formed.

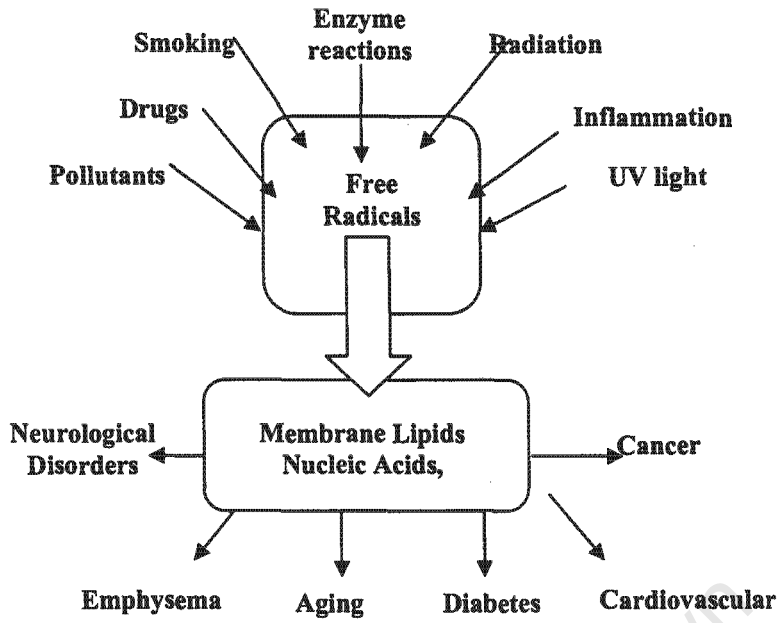


Figure 1-3. Free radical damage: A schematic diagram of endogenous and exogenous sources of free radicals in cells, adapted from Lachance *et al.*, (2001)

1.4.1 Free radical reactions in biological systems

Free radicals have damaging effects in biological systems. The formation of the hydroxide anion is due to the Fenton reaction;

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + {}^{\bullet}OH + OH^{-}$$

Hydrogen peroxide is formed in cell mitochondria, peroxisomes and microsomes, while intracellular Fe^{2+} is released as a response to the formation of the superoxide anion during stress conditions (Valko *et al.*, 2006). The hydroxyl radical is a very reactive chemical species (Pastor *et al.*, 2000) and when it is produced close to DNA, it can react with DNA bases or the deoxyribosyl backbone of DNA. This can result in strand breakage or the damaging of DNA bases (Valko *et al.*, 2006). Peroxyl radicals $ROO^{\bullet}$ (e.g dioxyl $HOO^{\bullet}$) are also highly reactive radicals and participate in diverse biological reactions. Lipid peroxidation is one such biological reaction, and peroxyl radicals are also involved in

DNA cleavage and in protein modifications. This radical also synergistically induces $O^{\cdot}_2{}^{-}$ DNA damage (Gutterage, 1995; Valko *et al.*, 2006).

1.4.2 Free radical oxidation pathway of oxidation

ROS initiate oxidation reactions, which continue in a destructive chain reaction until a chain breaker stops the cycle. Lipid oxidation takes place via a typical free radical oxidation pathway, involving three main mechanisms (Antolovich *et al.*, 2002), namely:

1. Non-enzymatic free radical mediated chain reactions;
2. Non-enzymatic free radical photo-oxidation; and
3. Enzymatic reactions

Non-enzymatic free radical chain reactions are the typical free radical route which can take place through H atom transfer, as is the case of lipid peroxidation. This takes place in four stages: initiation, propagation, branching and termination (Antolovich *et al.*, 2002). LH represents the lipid substrate molecule, and $R^{\bullet}$ the initiating oxidizing material. $L^{\bullet}$ is the resulting highly reactive radical.

Initiation

$$LH + R^{\bullet} \longrightarrow L^{\bullet} + RH$$

The process can be initiated by heat, light or by chemical means (Grey, 1986).

Propagation

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \longrightarrow L^{\bullet} + LOOH$$

The peroxyl radical $LOO^{\bullet}$ is formed from the reaction of $L^{\bullet}$ with oxygen. These radicals are the perpetuators of the chain reaction. They can further oxidise lipids, producing lipid hydroperoxides LOOH, which break down into various products such as alcohols, aldehydes and radicals such as the alkoxyl radical $LO^{\bullet}$.

Branching

$$LOOH \longrightarrow LO^{\bullet} + HO^{\bullet}$$

$$2LOOH \longrightarrow LOO^{\bullet} + LO^{\bullet} + H_2O$$

This step involves the breakdown of the lipid hydroperoxides and often involves transition metal ion catalysts, resulting in the production of the lipid peroxyl and alkolxyl radicals.

$$LOOH + M^{n+} + H^{+} \longrightarrow LO^{\bullet} + M^{(n+1)+} + H_2O$$

$$LOOH + M^{(n+1)+} + OH^{-} \longrightarrow LOO^{\bullet} + M^{n+} + H_2O$$

Termination

$$\left.\begin{array}{l} LO^{\bullet} + LO^{\bullet} \\ LOO^{\bullet} + LOO^{\bullet} \\ LO^{\bullet} + LOO^{\bullet} \end{array}\right\} \text{non-radical products}$$

The reaction between two free radicals results in the formation of non-radical products that terminate the chain reaction.

1.4.3 Antioxidants

Oxidative damage can be prevented by antioxidants. An antioxidant may be defined as "any substance which when present at low concentrations compared with those of the oxidisable substrate, significantly delays or inhibits oxidation of the substrate" (Halliwell, 1990; Halliwell and Gutteridge, 1989). Physiologically, oxidative stress is a result of an imbalance between endogenous antioxidants and cellular repair mechanisms operating in response to ROS. Enzymes such as superoxide dismutase, glutathione peroxidase and catalase form part of the cellular defence mechanism, together with small molecules such as vitamin E and serum albumins (Halliwell *et al.*, 1995; Antolovich *et al.*, 2002). Depending on their chemical nature, antioxidants can act in either hydrophobic or

hydrophilic cellular environments and some can work in both. Vitamin C, for example, is an aqueous antioxidant (present in the cell cytosol) whereas vitamin E is a lipophilic antioxidant (present in the cell membrane). α-lipoic acid is both water and fat-soluble and can therefore work in both phases. Antioxidants often form "antioxidant networks" in which some antioxidants regenerate other antioxidants, thereby recycling one another (Sies and Stahl, 2005). Vitamin E and C form such networks. The vitamin E component, α-tocopherol (α-TOH), is the most active known lipid-soluble chain breaking antioxidant known in human blood plasma (Burton and Ingold, 1986). The reaction rate of α-TOH with peroxyl radicals is approximately 10^6 M s^{-1}, which is greater than that of the lipid and peroxyl radical (Burton and Ingold, 1986). *In vivo,* α-TOH is regenerated by vitamin C from the α-tocopherol radical (α-$TO^{\bullet}$), providing a constant supply of the antioxidant (Tappel, 1968). This type of synergism is not uncommon amongst antioxidants and this aspect will be revisited in section 1.4.4.

Exogenous antioxidants, such as dietary antioxidants, have long been recognized for their health benefit in relieving oxidative stress. They are gaining attention as they may have applications in the pathology of cancer, malaria, rheumatoid arthritis, aging, and the prevention of chronic degenerative diseases through the amelioration of endogenous and exogenous cell sources of oxidation (Halliwell, 1987; Lachance *et al.*, 2001; Boreck, 2004).

1.4.3.1 Types of antioxidants

Antioxidants can delay the progression of free radical reactions. Primary or chain breaking antioxidants prevent the proliferation of free radical chain reactions, and secondary/preventative antioxidants inhibit the oxidation of the substrate (Jadhav *et al.*, 1996). Secondary antioxidants, also known as oxidation retarders (section 1.4.5), either physically remove the substrate or quench the mono-oxygen radical (Frankel and Meyer, 2000). Primary antioxidants, also called chain breaking antioxidants, usually intervene during the initiation or propagation step by reacting with the radicals (Jadhav *et al.*, 1996). Phenolic compounds are mainly thought to be primary antioxidants, and to play a

preventative role in oxidation. The mechanism by which they react with free radicals is either by H-atom transfer or electron transfer to free radicals. These are explained in more detail below.

1.4.3.2 Phenolic compound free radical quenching via H-atom transfer (HAT)

Phenolic antioxidants, by definition, contain at least one hydroxyl group on an aromatic ring (ArOH). They fall into the primary, chain-breaking category of antioxidants as they interrupt the chain reaction as illustrated in the equations below (Wright *et al.*, 2001; Antolovich *et al.*, 2002):

$$L^{\bullet} + ArOH \longrightarrow LH + ArO^{\bullet}$$

$$LO^{\bullet} + ArOH \longrightarrow LOH + ArO^{\bullet}$$

$$LOO^{\bullet} + ArOH \longrightarrow LOOH + ArO^{\bullet}$$

Antioxidant activity is inversely proportional to the ArO-H bond strength because the antioxidant free radical $ArO^{\bullet}$ must be stable. To be effective, a phenolic antioxidant should react slowly with the non-radical substrate LH, but rapidly with free radicals such as $LOO^{\bullet}$. The activation energy of the antioxidant free radical reaction decreases with its decreasing bond dissociation energy (BDE) thereby increasing the antioxidant efficiency. Bond dissociation energies have been used to predict the efficacy of phenolic antioxidants: the weaker the OH bond in ArO-H, the faster it reacts with free radicals (Wright *et al.*, 2001).

1.4.3.3 Phenolic compounds free radical quenching via single electron transfer (SET)

In this mechanism, the antioxidant quenches the free radical by donating an electron to it, which results in spontaneous and reversible deprotonation in solution, as illustrated below (Wright *et al.*, 2001):

$$LOO^{\bullet} + ArOH \longrightarrow LOO^{-} + ArOH^{+} \text{ (electron transfer)}$$

$$ArOH^{+} + H_2O \rightleftharpoons ArO^{\bullet} + H_3O^{+} \text{ (deprotonation equilibrium)}$$

$$LOO^{-} + H_3O^{+} \rightleftharpoons LOOH + H_2O \text{ (hydroperoxide formation)}$$

$$LOO^{\bullet} + ArOH \longrightarrow LOOH + ArO^{\bullet} \text{ Overall reaction}$$

The net reaction is the same as for the HAT mechanism. The radical cation $ArOH^{+}$ is reactive and may attack suitable substrates, as has been observed with aminophenol cation substitution on DNA (Berkowitz *et al.*, 1994). This makes the radicals potentially mutagenic.

The SET and HAT mechanisms take place simultaneously, but with different reaction rates. BDE (which correlates with HAT) and ionisation potentials (IP), which correlates with SET, have been shown to be important molecular characteristics affecting antioxidant activity, and have been used as primary indicators of antioxidant activity in solution (Wright *et al.*, 2001). Other factors, such as the presence of bulky groups near the OH group in an antioxidant, solvent characteristics (such as H-bonding), solubility and standard reduction potentials, all have an influence on the antioxidant thermodynamics, which in turn affect antioxidant activity (Burton and Ingold 1986).

1.4.4 Antioxidant activity *in vivo*

By definition, antioxidants are not restricted to any specific type of chemical compound and the question arises as to whether one can extrapolate what is observed *in vitro* to the *in vivo* environment, where antioxidants ultimately have their beneficial effect. It has been observed that this is not often the case, and there is a need for deeper understanding (Halliwell, 1990). Antioxidant activity *in vivo* is complex, involving antioxidant enzymes, iron binding and transport proteins, signal transduction and gene expression (Rice-Evans, 2004).

Natural antioxidants are often found as heterogeneous mixtures, of different compounds. Dietary antioxidants other than vitamin C, E and carotenoids, particularly phenolic compounds, are found in most plant material (fruits, vegetables and seeds) and plant products (olive oil, wine and tea) (Moure *et al.*, 2001). Phenolic compounds are plant secondary metabolites and are ubiquitous.

The effect of the different antioxidant groups on one another has been studied, and three types of antioxidant interactions have been characterised (Becker *et al.*, 2004):

- Synergism was defined by Uri (1961) as "the phenomenon in which a number of compounds, when present together, have a more pronounced effect than that which would be derived from a simple additivity concept".
- Antagonism is observed when the cumulative antioxidant effect of the antioxidants is less than the additive activity.
- Co-oxidation is when the total activity corresponds to the additive activity. The activity is neither higher nor lower but corresponds to computed activity based on theoretical data.

1.4.4.1 Antioxidant synergy

Not all antioxidants can scavenge different types of free radicals. Some antioxidants work well in certain environments such as lipophilic or aqueous environments, and thus combined use of antioxidants has been found to be beneficial (Bruun-Jensen *et al.*, 1996). Some antioxidants work in networks as described earlier, while others can recycle depleted antioxidants. Antioxidant synergism is important for the treatment of free radical-induced disease because as a result, lower antioxidant doses can be used for increased effectiveness (Moure *et al.*, 2001). According to Becker *et al.*, (2004) antioxidant synergism mechanisms involve:

- Regeneration: Where a more effective antioxidant regenerates a weaker antioxidant;
- Metal chelation: Where a metal chelating antioxidant (secondary antioxidant) chelates a metal such as iron, sparing other antioxidants such as chain breaking antioxidants (primary antioxidants);
- Solubility effects: Where antioxidants with different solubilities act in different phases, for example lipid- and water-soluble antioxidants; and
- Differing mechanistic effects: Where mixtures of antioxidants with different mechanisms of action, e.g. singlet oxygen quenchers and chain breakers with secondary antioxidants would have a broader range of antioxidant activity, and thus would be more effective.

1.4.5 Measurement of antioxidant activity

Antioxidant activity can be defined as the ability of a compound to significantly delay or inhibit oxidation of a substrate. The direct measurement of antioxidant activity *per se* is generally not possible, but the effects of the antioxidant on the degree of inhibition of oxidation can be measured (Antolovich *et al.*, 2002). The inhibition of ROS or the direct scavenging of free radicals would then be measured. Antioxidant activity can be obtained by measuring the decrease in oxidation or a decrease in the overall duration of oxidation.

The rate of oxidation is used as a measure for secondary antioxidants, and decrease in oxidation duration measurements are used for chain breaking antioxidants (primary antioxidants). Antioxidant assays are based on these parameters (Becker *et al.*, 2004). Retarders of oxidation are defined as "compounds that reduce the rate of oxidation without showing a distinct lag phase of oxidation" (Becker *et al.*, 2004). The differences in the oxidation profiles of chain breaking antioxidants and oxidation retarders are illustrated in Figure 1-4. Oxygen depletion in a closed food system is used as an example, in Figure1-4. Chain breaking antioxidants have a lag phase in their reaction pathway, whilst oxidation retarders do not.

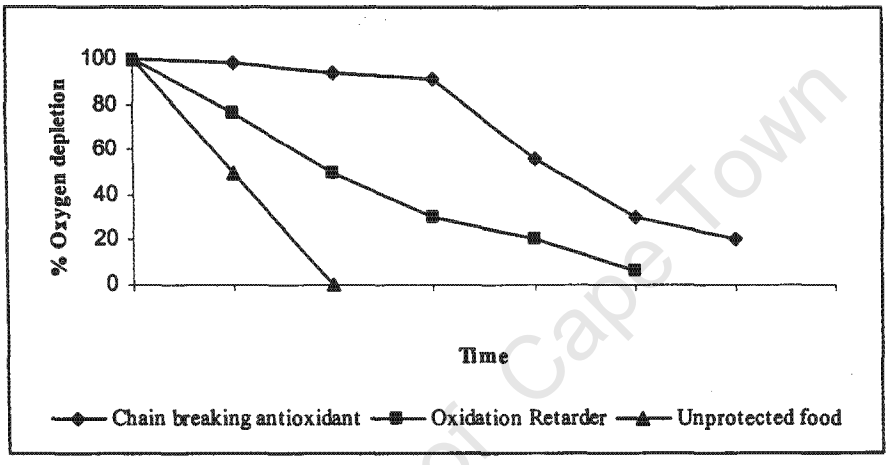


Figure 1-4. The differences in antioxidant activitity of oxidation retarders and chain breaking antioxidants. Oxidation retarders extend the time over which oxidation takes place, and chain breaking antioxidants delay the onset of oxidation. Oxygen depletion in a closed food system over time is shown (adapted from Becker *et al.*, 2004).

In investigations on antioxidants several factors are important:

- The identity, source and target of ROS. Some antioxidants may have a protective function against particular ROS, but a pro-oxidant effect on other biological molecules. For example, some polyphenolic compounds are terminators of free radical chain reactions but in the presence of metal catalysts such as iron, they behave as pro-oxidants (Valko *et al.*, 2006). Also, depending on the target of oxidation, the extent of inhibition of oxidation obtained may be different. Some antioxidants would exhibit greater antioxidant activity in, e.g. the prevention of

lipid peroxidation test than in other tests determined by the compatibility of the antioxidant with the type of ROS (Halliwell *et al.*, 1995; Antolovich *et al.*, 2002; Becker *et al.*, 2004).

- The presence of interacting components. Antioxidants interact as co-antioxidants, synergistically or antagonistically (see section 1.4.4). These interactions impact on the measured activity of a mixture, and it would be necessary to differentiate between antioxidant activity and antioxidant capacity. Antioxidant capacity is the result of all antioxidant reactions in a mixture and is due to the combined antioxidant activities. This point is particularly important in biological systems where there are many different types of antioxidants which can interact with one another.
- The antioxidant mechanism. It is necessary to assess the antioxidant activity in terms the antioxidant reaction mechanism. Whether it is a SET or HAT chain breaking antioxidant or a secondary antioxidant will have significance in terms of the assay chosen to assess its activity. It is also noteworthy that oxidation mechanisms may change under certain conditions such as changing temperature (Antolovich *et al.*, 2002).
- The test conditions. Some antioxidants are lipophilic and in hydrophilic test conditions, their activity might not be detected or may be under-represented.
- The antioxidant concentration. Antioxidant activity is often concentration-dependent: below a certain concentration, no antioxidant activity may be observed; above certain concentrations pro-oxidant activity may occur. As a result, it is necessary to perform *in vitro* antioxidant measurements at concentrations that are biologically (*in vivo*) relevant for the intended use of the antioxidant (Halliwell *et al.*, 1995).
- *In vivo* considerations. *In vivo,* certain factors are very important. Some compounds may have the ability to up-regulate endogenous antioxidants, e.g. by the up-regulation of genes encoding antioxidant enzymes (Halliwell, 1990). Bio-availability, absorption, metabolism and pharmacokinetics are important when assessing *in vivo* antioxidant activity (Moure *et al.*, 2001). Some antioxidants may not be soluble in biological systems and would not be available, as a result. Also,

the antioxidant needs to be at the site of oxidation. Transport of antioxidants into cells is not yet fully understood, but the sites of oxidation are often intracellular and antioxidant compounds would need to be transported into the cell to be effective.

The factors described above show the need for a multi-test approach when testing for antioxidant activity. One assay would probably not comprehensively represent the characteristics of an antioxidant. This is especially relevant in biological systems where there is interaction with various other antioxidants, and there are different sources and targets of ROS. Because of this, it is difficult to extrapolate *in vitro* antioxidant activity to the *in vivo* situation. Becker *et al.* (2004) have proposed an adapted scheme (Figure. 1-5) for antioxidant evaluation that takes into consideration the limitations discussed above.

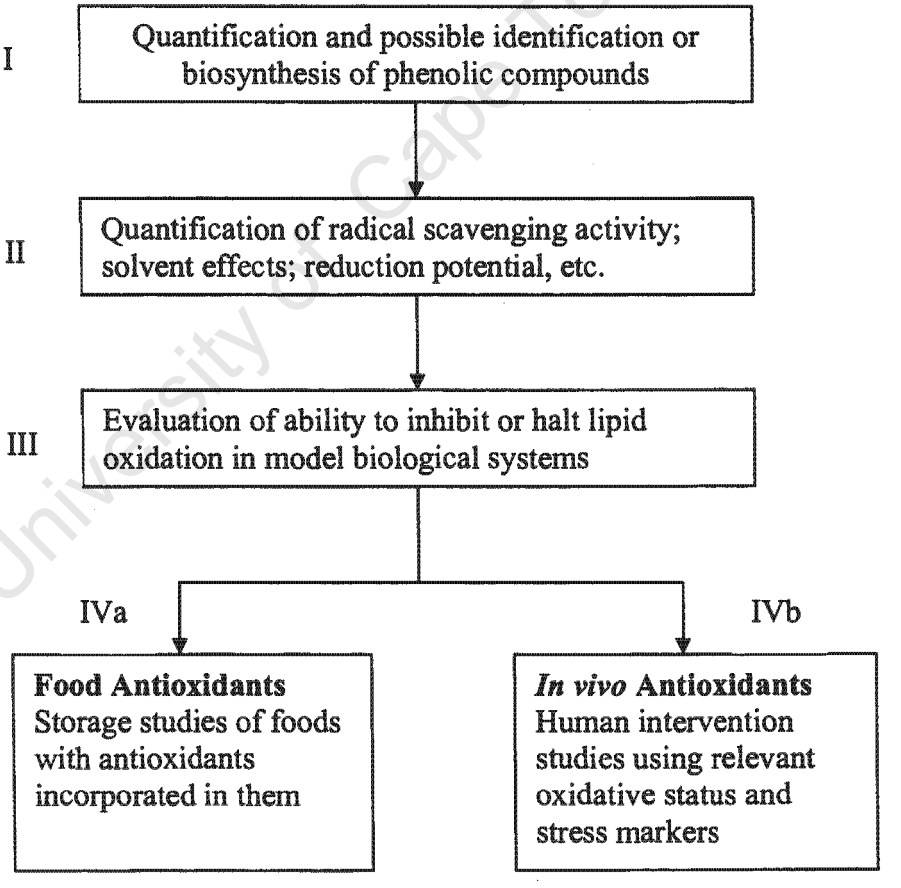


Figure 1-5. Assessment and application of antioxidant activity in foods and *in vivo* (adapted from Becker *et al.*, 2004).

1.5 Plant phenolic compounds as antioxidants

Plant phenolics are ubiquitous in plant material (Harborne, 1980), and since plants are a major component of the human diet, phenolics play a significant role in human diets. Phenolics are formed as part of plant secondary metabolism. Plant phenols have anticarcinogenic (Hertog *et al.,* 1993), anti-inflammatory, (Akhisa *et al.,* 2000) and ROS scavenging properties. They have also been found to increase the activity of phenolsulfotransferases that are important in detoxification (Yeh *et al.,* 2004) and to inhibit phospholipase A and cyclooxygenase. These properties are thought to be a result of their antioxidant properties (Yagi and Ohishi, 1979; Kikuzaki *et al.,* 2002). They have also been found to have vasodilatory, anti-bacterial, anti-allergic, immune-stimulating, and estrogenic effects (Rice-Evans *et al.*, 1996).

1.5.1 Health benefits of plant phenolic consumption

It has been shown that consumption of plant phenolic compounds reduces the risk of coronary heart disease (Duthie *et al.,* 2000). Higher than normal levels of cholesterol in low density lipoproteins (LDL) are found in sufferers of coronary heart disease (CHD) and atherosclerosis and oxidation of the lysine residues of apolipoprotein B, a main constituent of LDL, is thought to play a significant role in atherosclerosis. Phenols, particularly those with multiple hydroxyl groups, have been shown to reduce oxidation of lipid and LDL particles *in vitro* (Nardini *et al.,* 1995). Consumption of phenolic antioxidants is thought to be the explanation for the "French Paradox" where, in spite of high fat intake, certain French populations have very low incidence of coronary heart disease and related mortality.

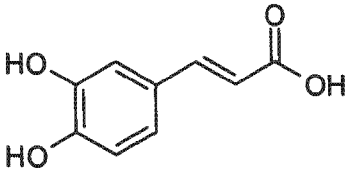
1.5.2 Chemical properties of plant phenols

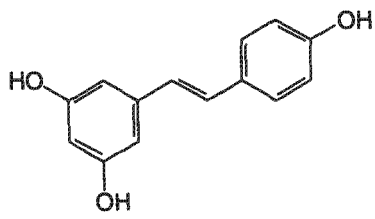
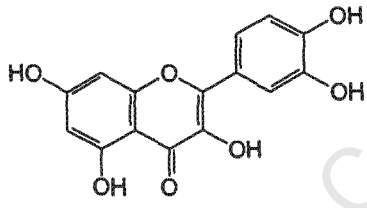
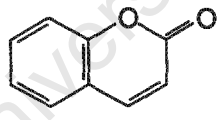
Several thousands of plant phenols have been identified (Harborne, 1980) and classified into various groups (shown in Table 1-2). They range from simple molecules such as

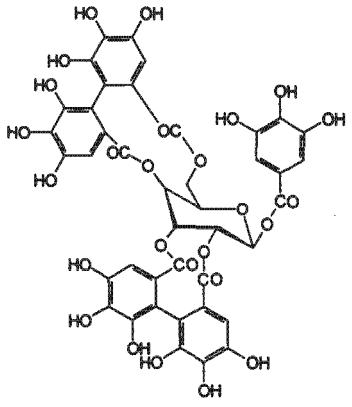
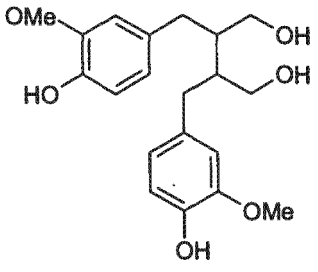
catechols to complex polymers (Parr and Bolwell, 2000). Their properties are largely related to their ability to generate stable free radicals due to the delocalisation that arises from the interaction of the hydroxyl group with the π-electrons of the benzene ring. These robust radicals are able to intervene in radical-mediated oxidation processes, which gives them their biological activity (Parr and Bolwell, 2000). They are also able to chelate metal ions, the extent depending on the presence of vicinal hydroxyl groups, e.g. in caffeic acid or quercetin (Morel *et al.,* 1993). This property is important biologically, as transition metal ions have pro-oxidant activity in the presence of hydrogen peroxide, which is a product of flavoprotein oxidases, which areprevalent in cells (Parr and Bolwell, 2000). Phenolic compounds also act as weak acids. Their hydroxyl groups are readily ionised due to the electron delocalisation, which influences their chemical reactivity. Their hydroxyl groups also easily form hydrogen bonds in the presence of H acceptors (Parr and Bolwell, 2000).

Phenolic compounds with antioxidant activity include the cinnamic acid derivatives such as ferulic acid, coumaric acid, catechol, isoeugenol and their derivatives and sterol derivatives such as γ–oryzanol, flavonoids, flavonols, anthocyanidins, stilbenes (e.g. resveratrol) and naringenin (Table1-2)

Table 1-2. Classification of phenolic compounds [Parr and Bolwell, (2000) and Scalbert and Williamson, (2000)].

Group	Example	Biological function /activity
Phenolic acids Hydroxycinnamoyl derivatives and benzoic acids	Caffeic acid 	Lignin monomers plant structural polymers

Stilbenes	Resveratrol 	Plant phytoalexins
Flavonoids; Isoflavones Flavones Flavonols Flavanols Anthocyanins Flavonons	Quercetin 	UV screens in plants Pigmentation Attractants Anticancer compounds Most abundant dietary phenols
Coumarins	Coumarin 	Plant signalling compounds Anticoagulants
Tannins (Polymeric phenolic compounds)	Casuarictin	Enzyme inhibitors Plant defence Plant pigments Leather formation

Ellagitannins Proanthocyanidins		
Lignans	Secoisolariciresinol 	Phytoestrogens Plant structural compounds

1.5.3 Biosynthesis of plant phenolic compounds

Metabolically, plant phenols are derived from the shikimate pathway and phenylpropanoid metabolism (Ryan and Robards, 1998). All major groups of phenolic acids are derived from the C3-C6 phenylpropanoids (ferulic acid, cinnamic acid, synaptic acid) except gallic acid derivatives as shown in Figure 1-6 below. The formation of different compounds is dependent on several factors such as environmental stimuli and condition or tissue type (Ryan *et al.*, 2002). Cellular phenolics are usually conjugated, which lessens their toxicity, enhances solubility, and may affect cellular localization and transport (Damtoft *et al.*, 1993).

1.5.4 Phenylpropanoid metabolism

Phenylalanine and tyrosine are formed via the shikimate pathway. Phenylalanine is the precursor of auxins (plant hormones) and is the initial substrate of phenylpropanoid metabolism (see Figure 1-6). Thus, phenylalanine may be involved in control of flux into the phenolics pathway (Ryan *et al.*, 2002). Phenylalanine ammonia lyase is the initial enzyme in the biosynthetic pathway, and initiates the biosynthesis of secondary compounds such as lignin and flavonoid pigments. Consequently, it is highly expressed at high levels in vascular tissue, where lignin accumulates, and in the epidermal tissues where flavonoid protection against UV radiation takes place (Ryan *et al.*, 2002). Phenolic acids such as ferulic and 4-coumaric acid control auxin biosynthesis and thus regulate plant growth (Lavee *et al.*, 1994).

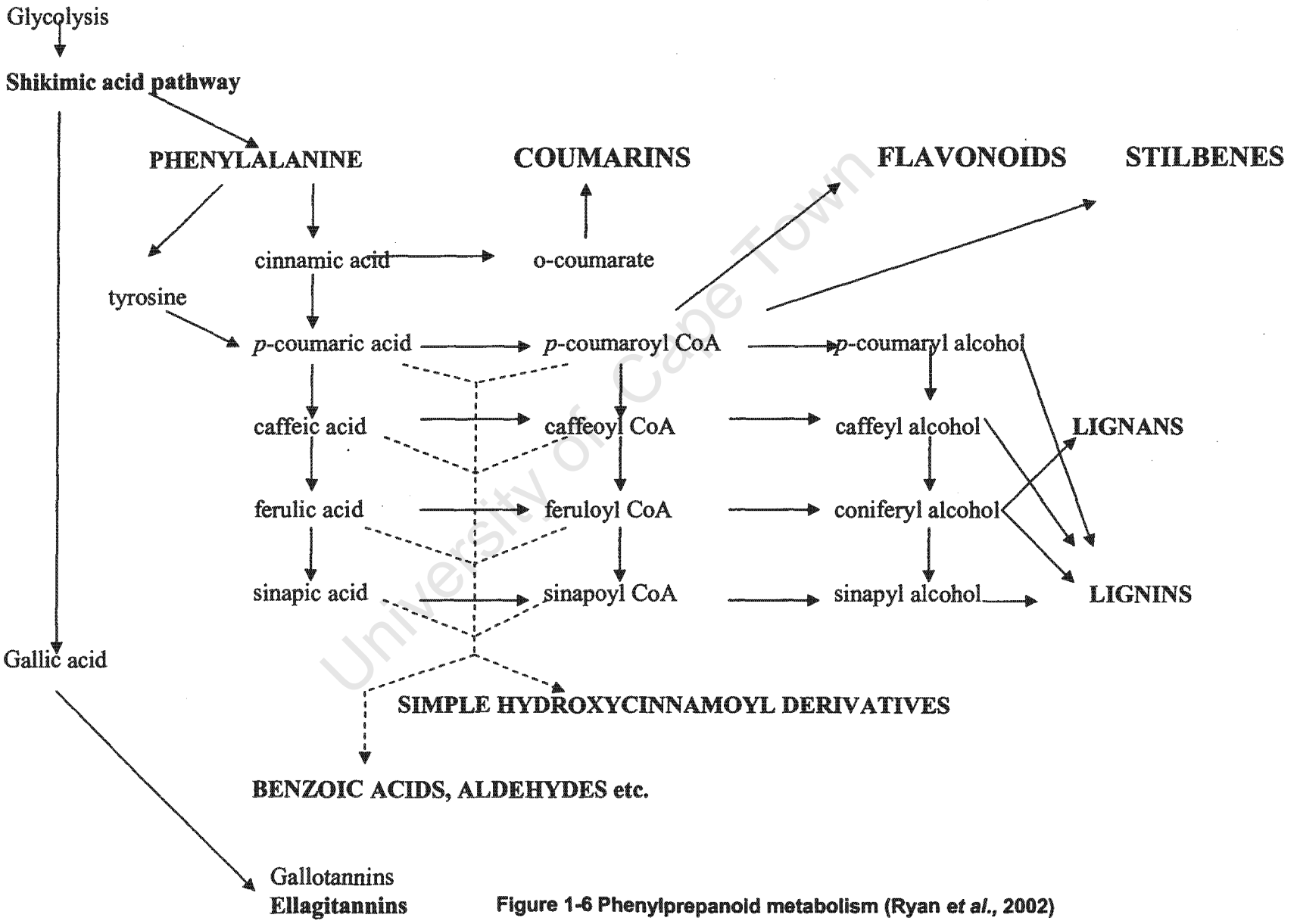


Figure 1-6 Phenylprepanoid metabolism (Ryan *et al.*, 2002)

1.5.5 Pro-oxidant activity of phenolic compounds

Compounds which exhibit pro-oxidant activity propagate or initiate free radical reactions (Young and Lowe, 2001). Many compounds, such as carotenoids and phenolic compounds, have been found to possess both antioxidant and pro-oxidant activity (Young and Lowe, 2001; Valko *et al* ., 2006). Complexes of Fe^{3+} and phenol are able to act as Fenton catalysts which are oxidising agents in themselves. Catecholic phenolics were found to catalyse Fe- dependent damage of DNA as well as damage of deoxyribose. However, these same phenolics were found to have antioxidative activity toward lipid peroxidation (Moran *et al.,* 1997). Pro-oxidant activity of polyphenols has been found under specific conditions (Valko *et al.,* 2006):

- when present in high concentrations;
- in the presence of redox active metals such as Fe^{2+}/Fe^{3+}; and
- high pH.

However, these conditions are relevant *in vitro* but do not necessarily apply *in vivo* where intercellular conditions are tightly regulated.

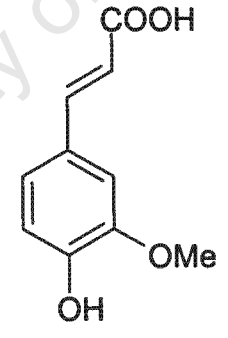
1.6 Vanillin and flavour compounds

Isolation of flavour compounds from plant material for use as flavourants and fragrances has been long established. For many years their extraction was the only method of obtaining them, till 1868 and 1874 when the first chemical preparations of coumarin and vanillin, respectively, were reported (Serra *et al.,* 2005). Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major organoleptic component of vanilla flavour the principal flavouring used in the food industry as well as in fragrance manufacture (Li and Rosazza, 2000). It is synthesised from kraft lignin at a market price between 100-270 times less than that of natural vanillin (Lomoscolo *et al.,* 1999; Muheim and Lerch, 1999). Therefore, opportunities are available for biocatalytic processes to produce "natural" vanillin and other components of natural vanilla such as vanillic acid, vanillyl alcohol and vanillin (Walton *et al.*, 2000). Several approaches have been developed and a

few processes have been patented. The most successful chemical feed-stocks have been eugenol and ferulic acid in production of vanillin using various microorganisms (Washisu *et al.,* 1993; Mulheim and Lerch, 1999). The pathways involved will be discussed under the section describing biotransformations of ferulic acid, which follows.

1.7 Ferulic acid

Ferulic acid is the common name for 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid. Alternative names include 3-methoxy-4-hydroxycinnamic acid, caffeic acid 3-methyl ester, and coniferic acid. The structure is illustrated in Figure 1-7. Ferulic acid is an extremely abundant plant component. It occurs primarily in seeds and leaves, in its free form and covalently linked to lignin and other biopolymers. It is a potent antioxidant compound which results from the formation of a resonance-stabilised phenoxy radical which is stabilised by extended side chain conjugation.



Ferulic acid

1

Figure 1-7. Chemical structure of Ferulic acid

Ferulic acid derives its name from the botanical name of the plant in which it was initially identified in 1866, *Ferula foetida*, an umbelliferous fennel-like plant (Hlasiwetz and Barth, 1866). It arises from the metabolism of tyrosine and phenylalanine. In plants it is hardly ever found in the free form (except in seeds and leaves), but occurs mainly as an ester, in plant cell walls (Graf, 1992). Ferulic acid has also been identified as a lipid moiety; structural tissues have been identified that contain long chain n-alkyl ferulates, ω-hydroxy fatty acid ferulates and sterol ferulates. Sterol ferulates were first discovered in the case of γ- oryzanol, an extract of rice oil which has great commercial and pharmaceutical significance discussed in more detail later (Graf, 1992).

Ferulic acid is a strong dibasic acid. The first proton dissociation produces the carboxylate anion, while the second dissociation generates a phenolate anion (Graf, 1992). The high degree of resonance stability of the phenolate anion across the entire conjugated molecule markedly increases its acidity relative to similar phenolic acids (Kenttamaa *et al.*, 1970). Oxidative degradation of ferulic acid leads to the production of vanillin and guaiacol (see Section 1-9). Other modifications of the propenoic acid side chain may produce additional classes of phenylpropanoid structures, such as flavonoids (Graf, 1992).

Ferulic acid and its derivatives have been shown to be potent natural antioxidants, and the major biological activity of ferulic acid is thought to be antioxidant activity, associated with other phenolic compounds (Graf, 1992). Plant allelopathic regulation and protection mechanisms have been shown to be related to ferulic acid content (Graf, 1995). The following functions in plants have been ascribed to ferulic acid:

- Cell wall strengthening. Ferulic acid strengthens the cell wall and endows structural rigidity by cross linking hydrolytic enzyme-susceptible components during germination (Tan *et al.*, 1992).
- Plant growth regulation. It inhibits the growth of competing plants. High levels of this compound also inhibit root growth and the development of the root system architecture. High levels of ferulic acid appear to be inversely related to mineral

and water uptake, hyphal elongation and the hydrolytic enzyme activities of germinating seeds (Rosazza *et al.*, 1995).

- Protection. Ferulic acid and its derivatives confer protection to plants against avian, insect, viral and fungal invasion (Rosazza *et al.*, 1995). The UV radiation absorbing properties of ferulic acid confer additional protection to plants.

1.8 Microbial transformations of ferulic acid

Microbial biotransformations of ferulic acid have been well documented and reviewed by Rosazza *et al* (1995). Ferulic acid biotransformations have been widely utilised, for example in the synthesis of L-phenylalanine analogs and the synthesis of aromatic flavour compounds (Renard *et al.*, 1992). The major pathway of microbial transformations of ferulic acid is shown below in Figure 1-8.

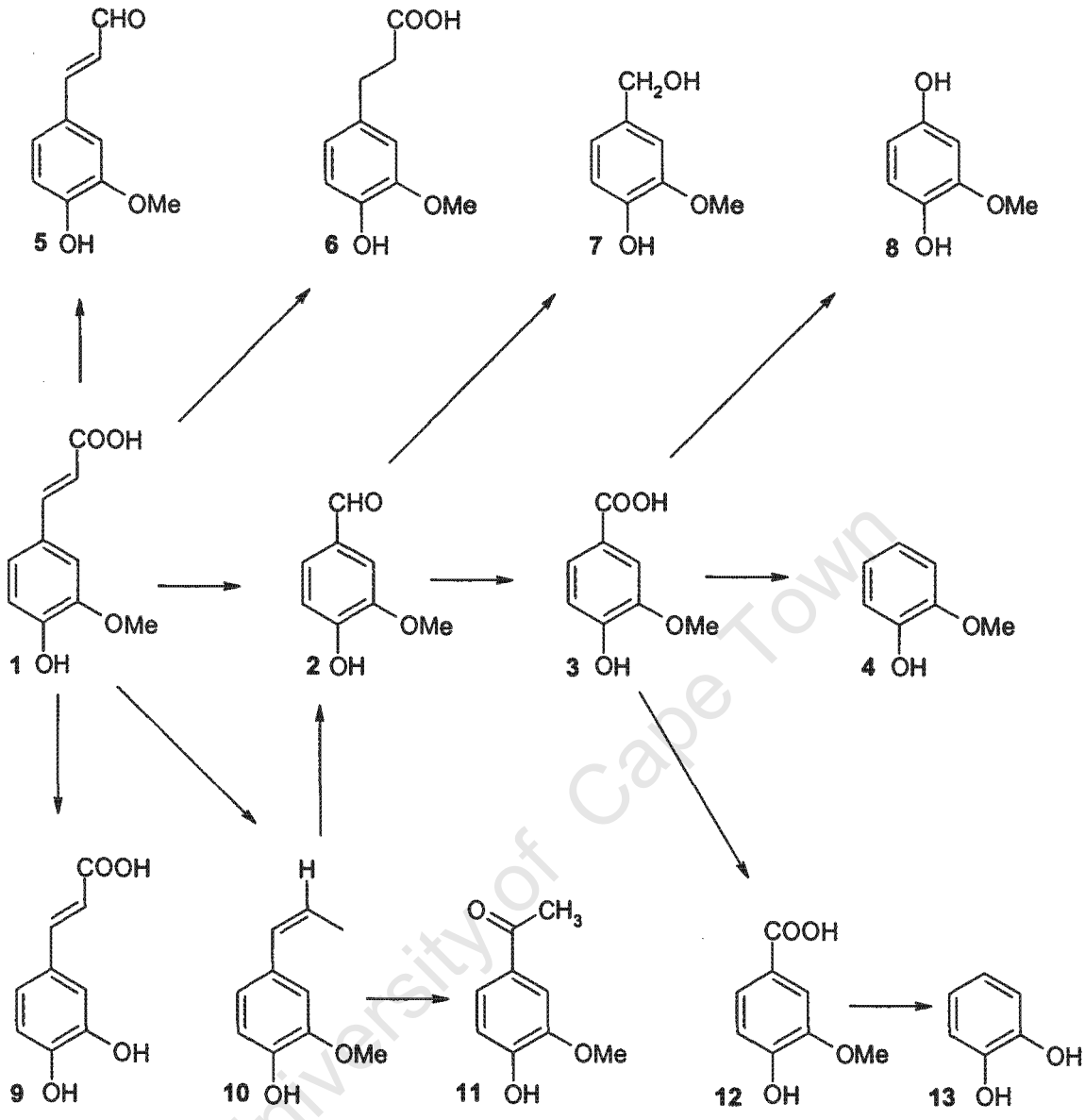


Figure 1-8. Microbial transformations of ferulic acid (adapted from Rosazza *et al.*, 1995).

The names of the compounds numbered 1-13 are as follows: **1:** Ferulic acid; **2:** Vanillin; **3:** Vanillic acid; **4:** Guaiacol; **5:** Coniferaldehyde; **6:** 4- hydroxy-3-methoxyphenylpropionic acid/dihydroferulic acid; **7:** Vanillyl alcohol; **8:** methoxyhydroquinone; **9:** Caffeic acid; **10:** 4-vinylguaiacol; **11:** 1-(4-hydroxy-3-methoxyphenyl)-ethanone; **12:**Protocatechuic acid; and **13:** 1,2 benzenediol.

1.9 Microbial degradation of ferulic acid

Much research has been conducted with the aim of elucidating the microbial degradation of ferulic acid, as many of the intermediates are industrially important compounds. This would allow for metabolic engineering of micro-organisms in order to produce them more efficiently. The degradation of ferulic acid has been categorized into four pathways, according to the initial reactions: β-oxidation, non-β-oxidation, side chain reduction and non-oxidative decarboxylation (Rosazza *et al.*, 1995):

- β-Oxidation of ferulic acid. This pathway is proposed to be analogous to the β-oxidation of fatty acids. It includes thiolytic cleavage of 4-hydroxy-3-methoxyphenol-β-ketopropionyl-coenzyme A (CoA) to yield acetyl-CoA and vanillyl-CoA (Figure. 1-9). It is postulated that the product is then cleaved by a β-ketoacyl-CoA thiolase. However, no experimental work has been done to support this theory (Peng *et al.*, 2003).
- Non β-oxidation of ferulic acid. This pathway has been demonstrated in *Pseudomonas fluorescens* and several other bacteria (Gasson *et al.*, 1998; Overhage *et al.*, 1999). Ferulic acid is converted to feruloyl-CoA, which undergoes side chain cleavage by hydration to form vanillin and acetyl-CoA (Figure. 1-9).
- Non-oxidative decarboxylation. This pathway has also been observed in many bacteria. The mechanism involves the loss of one carbon from the side chain of ferulic acid, catalysed by a non-oxidative decarboxylase, and the product is 4-vinylguaiacol. This compound itself has significant value and has been transformed into vanillic acid via vanillin (Rosazza *et al.*, 1995).
- Side chain reduction. This degradation reaction has been found to take place under aerobic and anaerobic conditions. In this case ferulic acid is reduced to 4-hydroxy-3-methoxyphenylpropionic acid, which can be further converted into vanillic acid via homovanillic acid (Ohmiya *et al.*, 1986).
- Other proposed pathways. Ferulic acid has been transformed into vanillic acid via vanillin, preceded by 4-hydroxy-3-methoxyphenyl-β-hydroxypropionic acid (Figure 1-10) (Peng *et al.*, 2003).

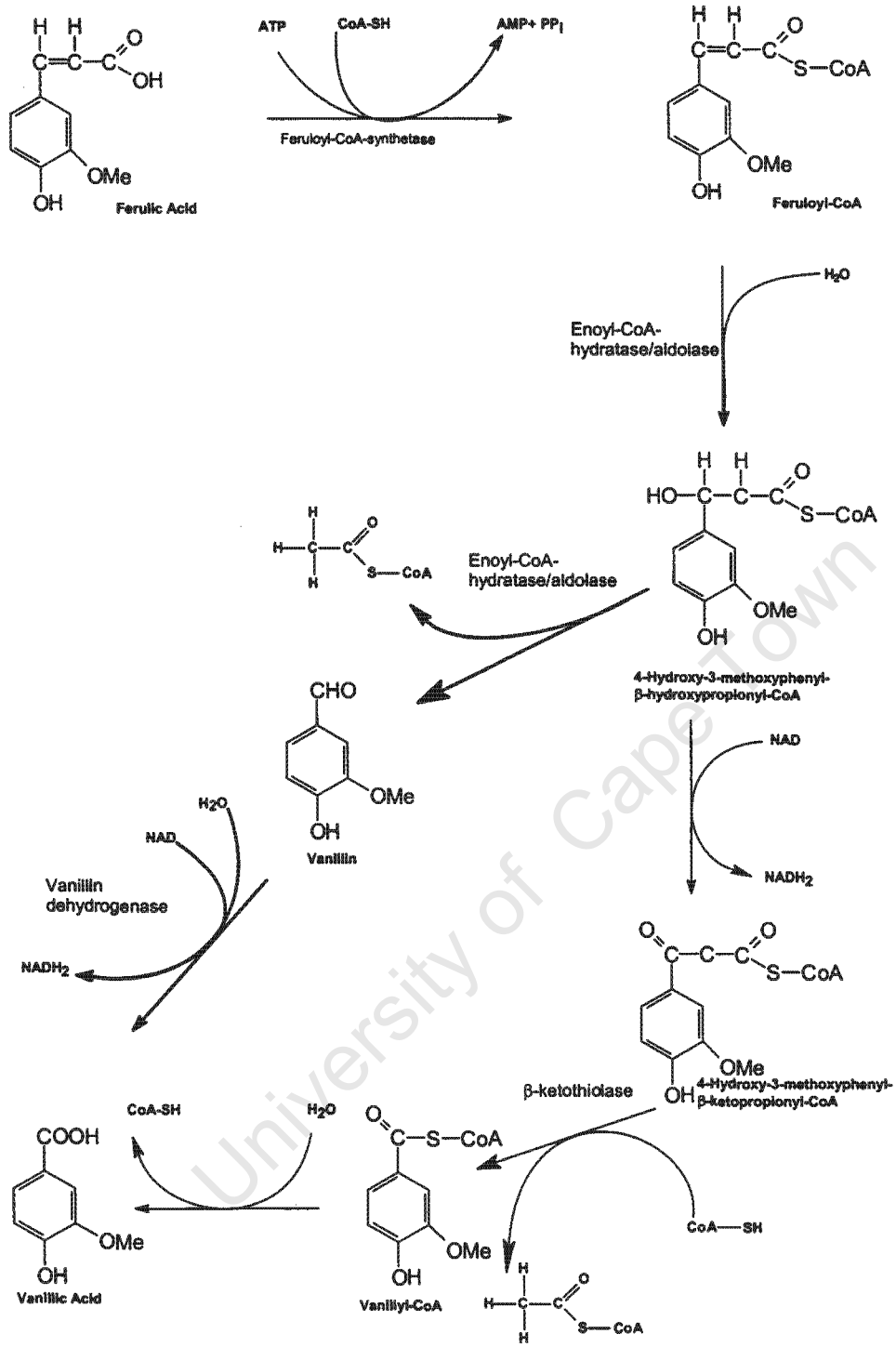


Figure 1-9. Non β-oxidation pathway of ferulic acid degradation as proposed in *Pseudomonas* sp. strain HR199. The fine lines show β-oxidation analogues to those of fatty acid catabolism. Heavy arrows show ferulic acid degradation via vanillin (adapted from Overhage *et al.*, 1999).

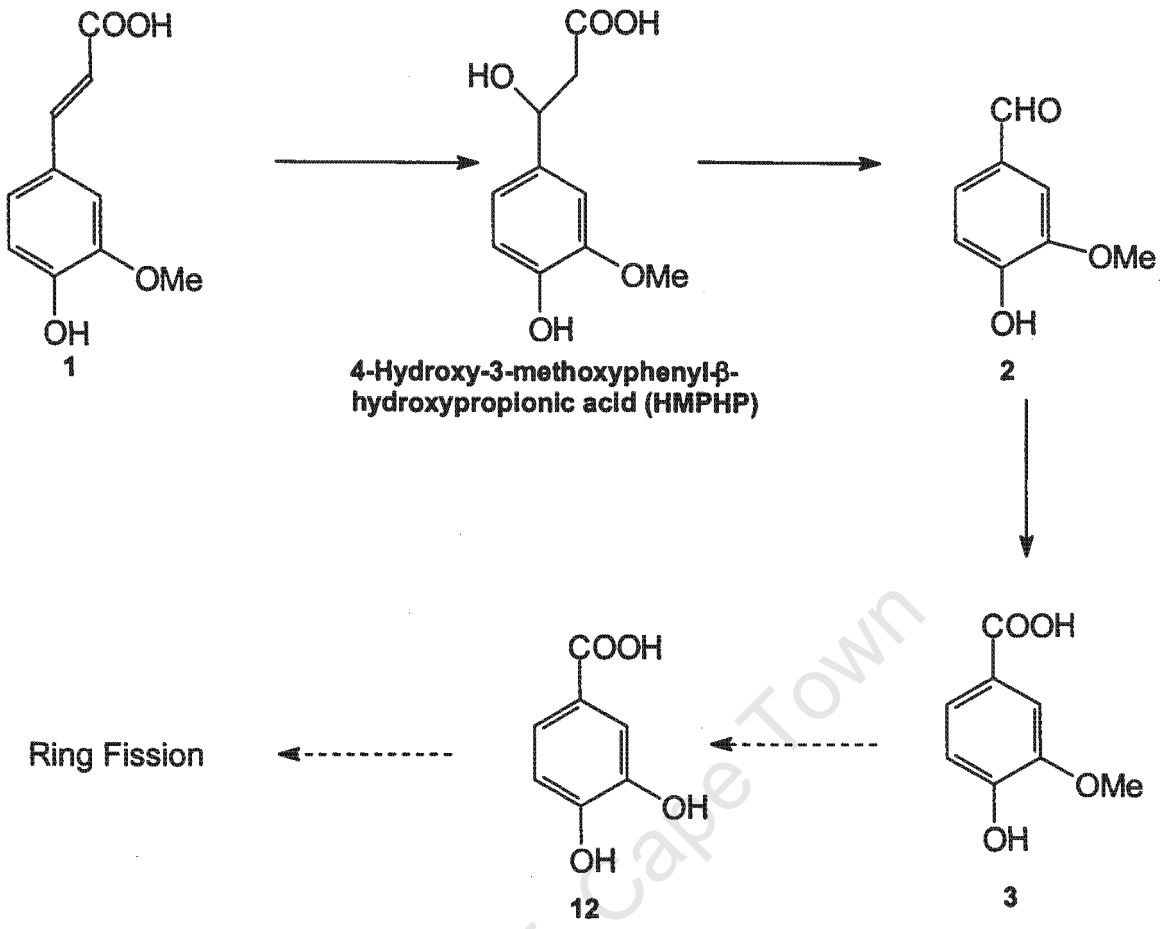


Figure 1-10. Pathway for the thermophilic biodegradation of ferulic acid by Bacillus isolates. The dotted arrows represent unidentified steps in the pathway (adapted from Peng et al., 2003).

1.10 Examples of biocatalytic processes involving ferulic acid

Ferulic acid has been used in many biotransformations, some which are discussed below.

1.10.1 Synthesis of vinyl styrenes

One of the oldest biotransformations of cinnamic acid derivatives is the formation of styrenes. Reports of fungal styrene synthesis reactions date back as far as 1906 (Hertzog and Ripke, 1906) and 1956 (Chen and Peppler, 1956). Styrene formation is the most

common microbial reaction that ferulic acid undergoes (Rosazza *et al.*, 1995). The reaction occurs rapidly and in high yield, but the mechanism is not well understood (Rosazza *et al.*, 1995).

1.10.1.1 Styrene production using plant cells

The common chemical method for the synthesis of styrenes is the decarboxylation of *trans*-cinnamic acids, which include ferulic acid. The reactions are normally carried out by heating under reflux at 200-300°C. The biotransformation of ferulic acid has the advantage of taking place under mild reaction conditions. Ferulic acid was dissolved in plant cell culture medium and 4-hydroxy-3-methoxy-styrene (4-vinylguaiacol) was produced quantitatively. Freely suspended *Catharanthus roseus* cells, in the stationary phase, and homogenised plant cell culture in 0.1 M phosphate buffer pH 6.4, were used to perform the decarboxylation (Takemoto and Achiwa, 1999). The reaction is shown below in Figure 1-11.

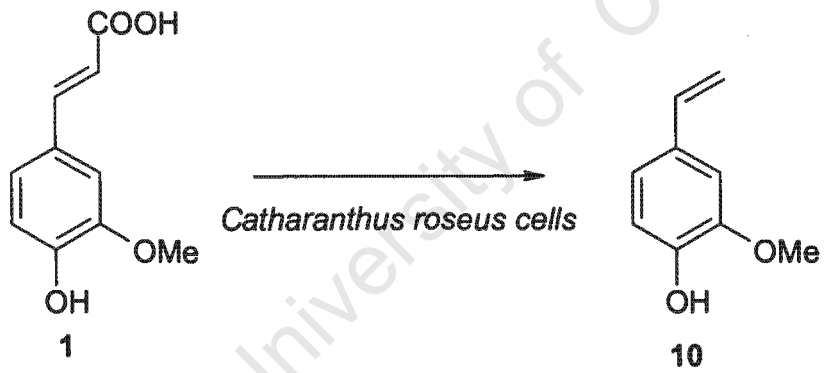


Figure 1-11. Production of 4-vinylguaiacol from ferulic acid catalysed by *Cantharanthus roseus* cells (Takemoto and Achiwa, 1999).

1.10.2 Synthesis of enantiopure phenyl-coumarans

The phenyl-coumaran skeleton structure, found in neolignans, is ubiquitous in the plant kingdom, including such diverse sources as ferns, tobacco, and loblolly pine (Satake *et al.*, 1978; Binns *et al.*, 1987; Nose *et al.*, 1995), which suggests their role in a universal

chemical defence system (Asakawa, 1998). For example, the dilignol 3'-4-di-*O*-methylcedrusin is a wound-healing agent and an inhibitor of thymidine incorporation in endothelial cells (Pieters *et al.*,1993) and dehydrodiconiferyl alcohol plays a role in promoting cell division in tobacco tissue cultures (Teutonico *et al.*, 1991). Thus, these compounds have potentially important pharmacological applications. Orlandi *et al.* (2001) synthesised enantiopure phenyl-coumarans by means of stereoselective phenol coupling oxidation of enantiopure ferulic acid derivatives catalysed by the enzyme horseradish peroxidase (HRP). In one approach, a ferulic acid amidic derivative was synthesised during the condensation reaction between ferulic acid **(1)** and ethyl (*S*)-alaninate **(14)** in the presence of dicyclohexylcarbodiimide (DCC). An HRP-catalysed oxidative phenol coupling was then performed in a dioxane-aqueous buffer at pH 3, obtaining a mixture of two **(15)** diastereoismers with 70% yield. The reaction is summarised in Figure 1-12.

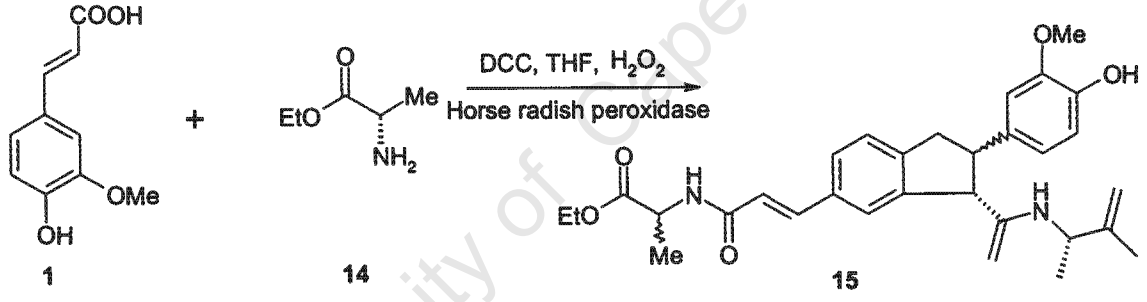


Figure 1-12. Biocatalytic production of intermediate diastereoisomers for the synthesis of dehydrodiconiferyl alcohol (Orlandi *et al.*, 2001).

The hydrolysis of product **(15)** with $LiOH/H_2O_2$ in THF with subsequent treatment with diazomethane, resulted in the synthesis of a dimethylester **(16)**, Figure 1-13.

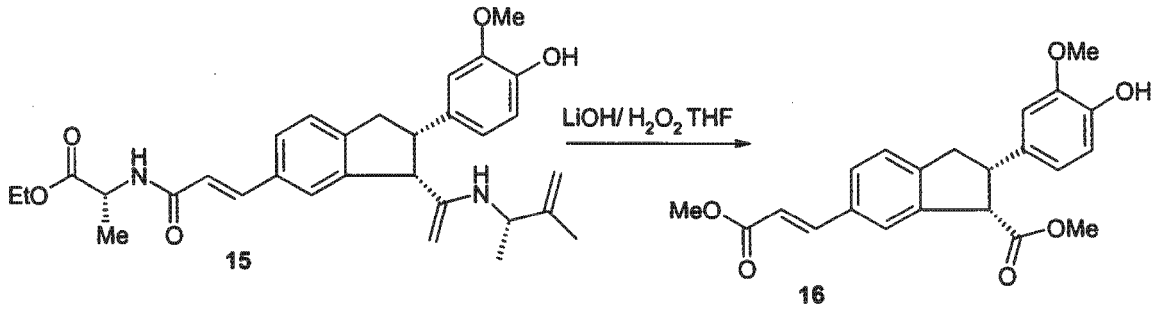


Figure 1-13. Chemical production of intermediate dehydrodiconiferyl dimethylester for the synthesis of dehydrodiconiferyl alcohol (Orlandi *et al.*, 2001).

The dimethylester was then reduced with $LiBH_4$ to optically pure dehydrodiconiferyl alcohol DDA (17) Figure 1-14.

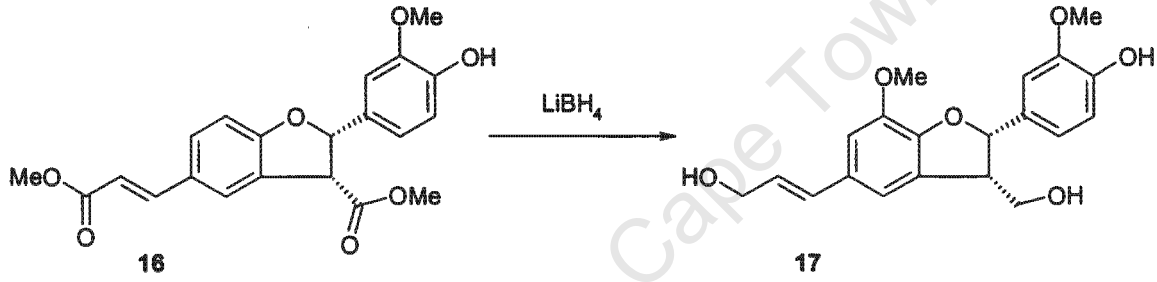


Figure 1-14. Final step in the chemo-biocatalytic manufacture of dihydrodiconiferyl alcohol (Orlandi *et al.*, 2001).

1.10.3 Production of vanillin

A wide range of micro-organisms can degrade ferulic acid to various products, including the much sought-after flavour compound, vanillin (section 1.6). Two examples that were relatively successful are discussed below, one in which a two-stage biotransformation involving filamentous fungi was used and another in which a yeast was used.

1.10.3.1 Production of vanillin by a two stage biotransformation using filamentous fungi

The degradation of ferulic acid by most microbes usually results in the synthesis of vanillic acid and vanillin as intermediates. Vanillin is the higher value compound, and a two stage process was developed to increase its yield.

In the first stage, *Aspergillus niger* was used to convert ferulic acid to vanillic acid. A laccase-deficient mutant strain of *Pycnoporus cinnabarinus* then reduced the acid to vanillin (Lesage-Meessen *et al.*, 1996) (Figure 1-15) the laccase-deficient strain being necessary due to the polymerising effect this enzyme has on phenols. Laccase was found to be associated with the polymerisation of ferulic acid and the loss of phenolic monomers from the culture medium (Lomoscolo *et al.*, 1999). The process yield was increased to 500mg vanillin per litre by adding cellobiose to the *Pycnoporus cinnabarinus* fermentation (Lesage-Meesen *et al.*,1997)

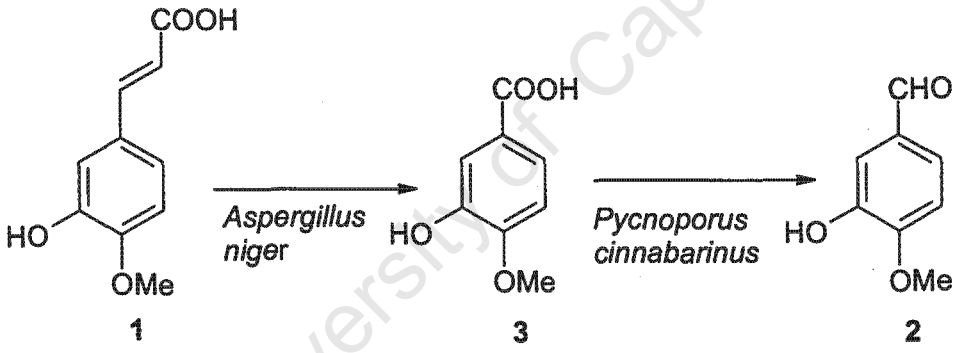


Figure 1-15. Two stage biotransformation of ferulic acid to vanillin using two different filamentous fungi.

1.10.3.2 Vanillin production from glucose

A genetically altered *E. coli* strain was used for this biotransformation. A fed batch fermentation was used to generate vanillin via the shikimate pathway. The *E.coli* strain used carried 3 mutated genes: 1) shikimate dehydrogenase; 2) 3-dehydroquinate synthase; and 3) dehydroshikimate dehydratase. The end product was a strain that generated an

excess of 3-dehydroshikimate which was blocked to further conversion to shikimate, producing protocatechuate instead. This product was then methylated to produce vanillate. The vanillate was then extracted in an acidified broth using ethyl acetate, and was reacted with an aryl dehydrogenase from *Neurospora crassa* (Li and Frost, 1998). The process has limitations such as low yields, but also has the advantage of using glucose, which is abundant and inexpensive. Figure 1-16 illustrates this reaction.

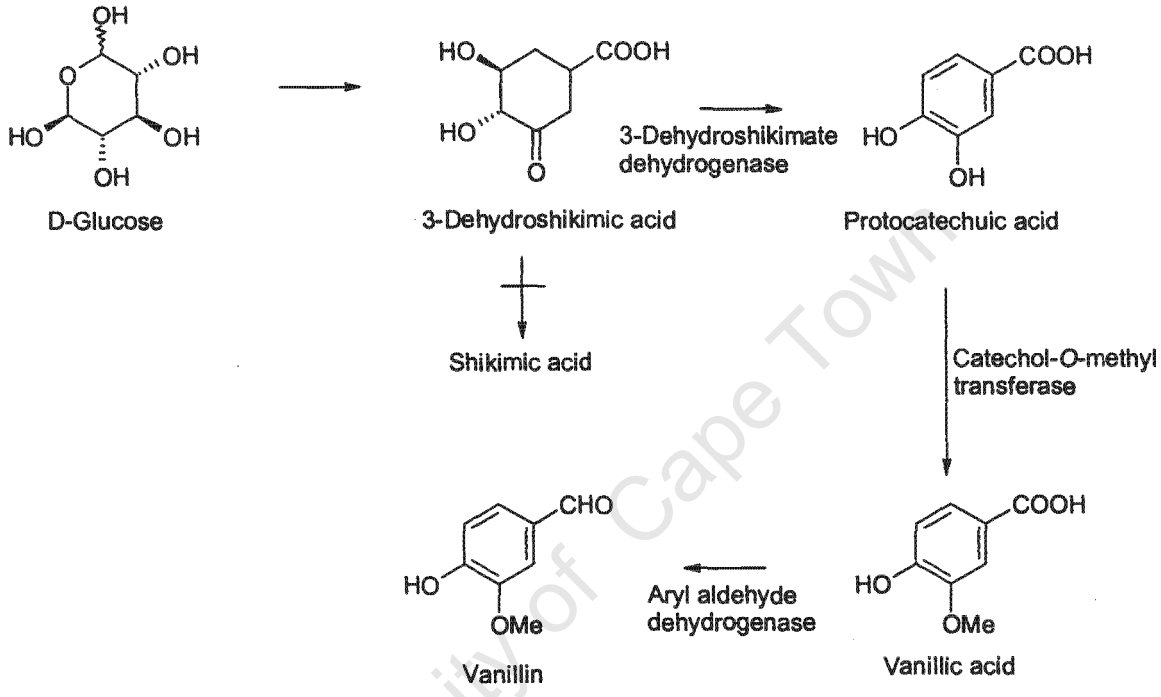


Figure 1-16. Biotransformation of glucose to vanillin. Recombinant *E. coli* strain KL7 converts glucose to vanillic acid which is then reduced *in vitro* to vanillin using an aryl aldehyde dehydrogenase from *Neurospora crassa* (Li and Frost, 1998).

However, all of these methods have failed to provide industrially viable processes, with most of the vanillin yields being very low. A more successful two stage process has been developed in which the fungus, *Aspergillus niger,* and the white rot fungus *P. cinnabarinus,* transformed ferulic acid to vanillin (Lesage-Meesen *et al.,* 1996).

1.11 White rot fungi

The white rot fungi are the only organisms known to completely metabolise lignin to carbon dioxide and water (Kirk and Farrell, 1987). Ferulic acid and other phenolic compounds are monomers of lignin; hence, white rot fungi and their enzymes are relevant in considering biotransformations of ferulic acid (ten Have and Teunissen, 2001). The white rot fungi derive their name from the bleaching effect that their substrate (wood) undergoes upon degradation. They are a physiological, rather than taxonomic grouping, with most of them being *Basidomycetes*, although a few genera within *Xylariaceae* are also capable of white rot decay (Eaton and Hale, 1993).

Lignin is a heterogeneous polymer that occurs in woody and vascular tissues. Random polymerisation reactions of *p*-coumaryl alcohol, 3-(4-hydroxyphenyl)propen-1-ol radicals and their methoxy-substituted derivatives results in formation of lignin. It forms a matrix surrounding the cellulose in woody cell walls, which protects the holocellulose from microbial degradation (ten Have and Teunissen, 2001). White rot fungi do not use the lignin as their energy source; in fact they degrade it to access the holocellulose it protects, which is their carbon and energy source (Leatham, 1986). Studies of the fungus *Phaneorchaete chrysosporium* showed that lignin degradation is stimulated during secondary metabolism when there is a deficiency of nutrient nitrogen, carbon or sulphur (ten Have and Teunissen, 2001). However, some species of fungi, such as *Trametes versicolor,* have emerged that are not regulated by nitrogen. The lignolytic activities of these fungal species were not repressed by addition of organic NH_4^+ nor by addition of L-amino acids (Collins and Dobson, 1995).

The lignin-degrading system of white rot fungi is composed of a group of extracellular enzymes including ligninolytic peroxidases, H_2O_2 generating oxidases, and low molecular weight cofactors (Kirk and Farrell, 1987). Three enzymes have been identified as being essential for lignin degradation and are often referred to as lignin modifying enzymes, LME's. The three enzymes comprise lignin peroxidase, Mn-dependent peroxidase, and a copper containing phenoloxidase, laccase. The redox mediator veratryl

alcohol is oxidised by lignin peroxidase, in the presence of endogenous H_2O_2, and the oxidised mediator then oxidises non-phenolic aromatic nuclei in lignin, generating aryl cation radicals. These then degrade non-enzymatically to aromatic and aliphatic products, which are mineralised intracellularly. The radicals generated can then carry out a range of reactions such as carbon-carbon bond cleavage, hydroxylation, phenol dimerisation/polymerisation, and methylation. Laccase also generates radicals from a redox mediator, in an H_2O_2 independent reaction.

Phenolic lignin components are also oxidised by Mn^{3+}, such as Mn^{3+} oxalate, or some other dicarboxylate which have been generated from the MnP-catalysed H_2O_2-dependent oxidation of Mn^{2+}.

1.12 Laccases

The enzyme laccase (*p*-diphenol: dioxygen oxidoreductase; EC 1.10.3.2), was first discovered from the Japanese lacquer tree, *Rhus vernicifera,* in 1883 (Yoshida, 1883). Laccase is widely distributed in plants and plays a role in lignin synthesis and is implicated in wound response (Lewis *et al.,* 1998). The same enzyme is involved in the phytopathogenicity of several fungi (Nicole, 1982). It is present in a range of organisms, ranging from bacteria (Givaudan *et al.,* 1993) to insects (Gianfreda *et al.,* 1999).

Laccase belongs to the well-defined group of multicopper oxidases which also includes enzymes such as ascorbate peroxidase and ceruloplasmin. Functionally, all enzymes in this group couple the four-electron reduction of dioxygen to water with the oxidation of a substrate (Solomon *et al.,* 1996). In the case of laccase, the substrate is oxidised by one electron. Laccases exhibit a wide range of substrate specificity for phenolics, which varies from one laccase to another, and they have overlapping substrate range with the monophenol mono-oxygenase, tyrosinase, but does not oxidise tyrosine as the latter does (Thurston, 1994). Laccase model reactions are shown below in Figure 1-17.

Laccase has also been implicated in lignin polymerisation. This ability to polymerise polyphenols, methoxy-substituted phenols, diamines and other compounds has resulted in laccase being used in bioremediation of wastewaters high in phenolic content and in the paper and pulp industry (Thurston, 1994; Solomon *et al.*, 1996).

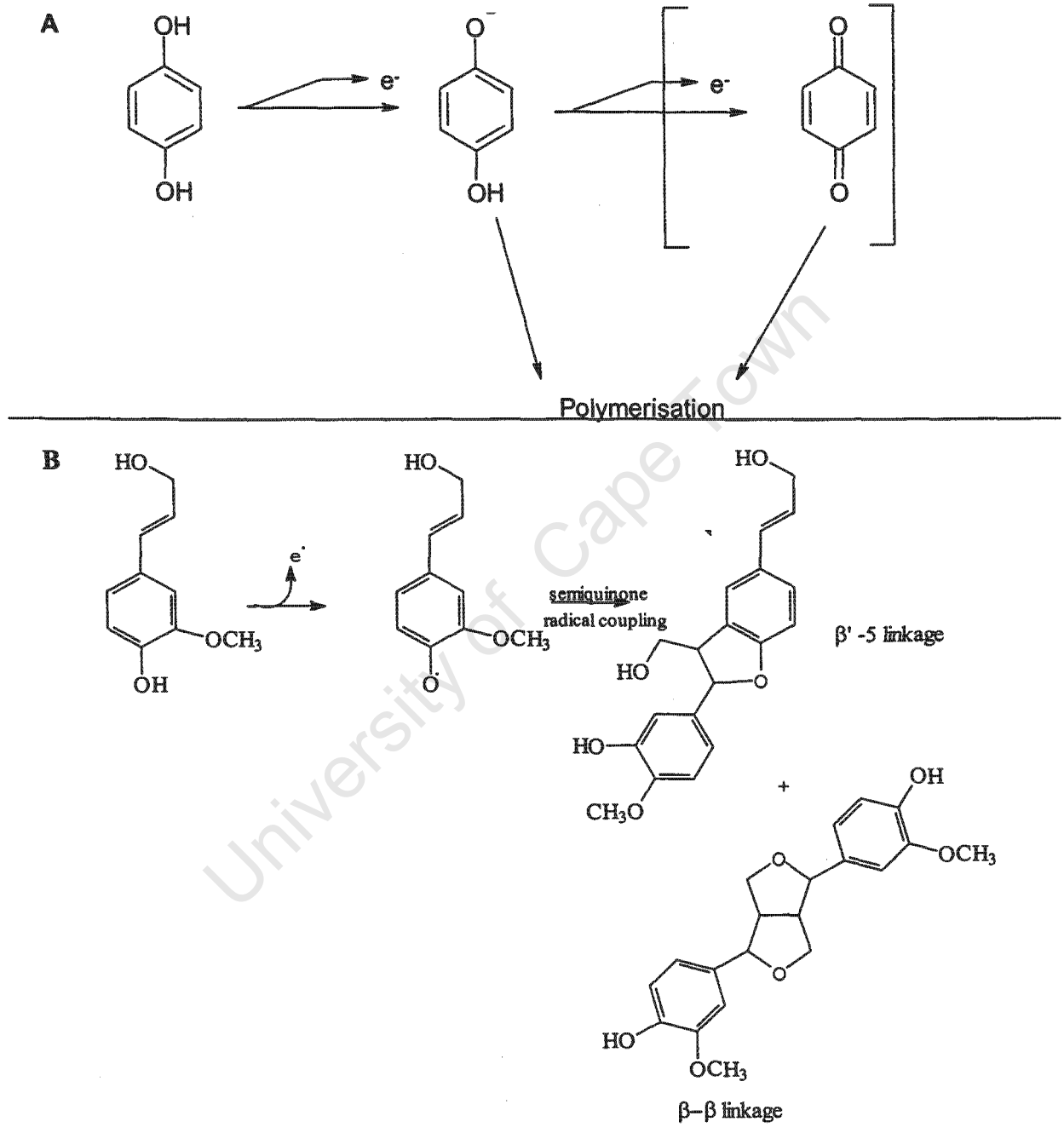


Figure 1-17. Typical laccase reactions: (A) shows the one electron oxidation of hydroquinone (a diphenol), to form an oxygen centred free radical. Following a second

enzyme-catalysed or spontaneous reaction, the free radical is converted to quinone. Quinone and free radicals undergo polymerisation. (B) A typical plant laccase reaction showing the oxidation of coniferyl aldehyde to two types of lignin dimer linkages (Figure A adapted from Thurston, 1994; B adapted from Solomons *et. al.*, 1996)

1.12.1 Structure of laccases

Laccases contain 4 copper ligands in their active site which are involved in intramolecular electron transfer, shuttling electrons from the substrate to the trinuclear cluster, which is the site of dioxygen binding and reduction (Lowery *et al.*, 1993; Solomons *et al.*, 1996) (Figure 1-18).

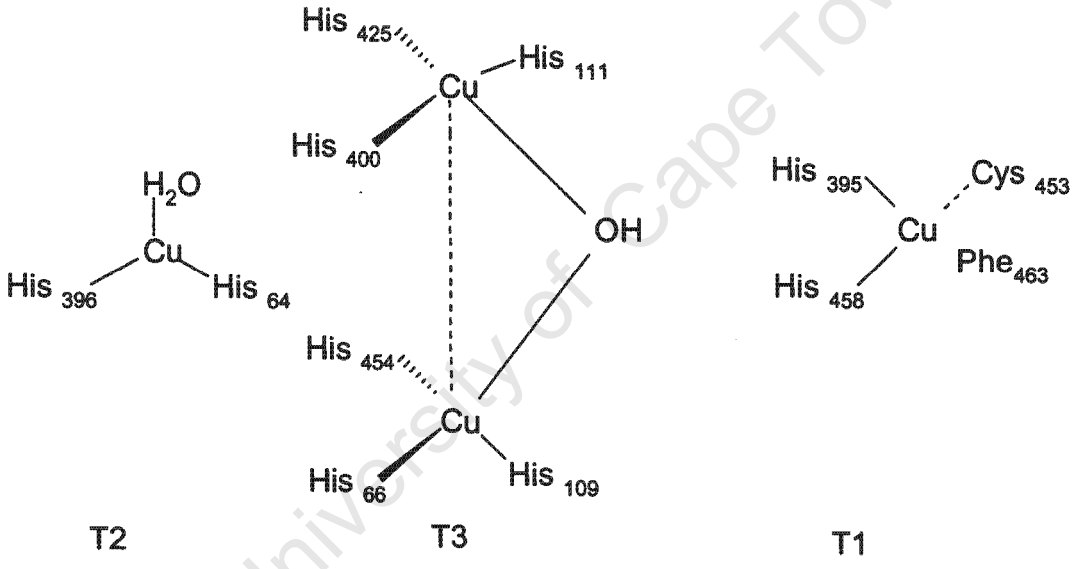


Figure 1-18. Model of the structure of *Trametes versicolor* laccase active site showing the four copper atoms. Type 1 (T1) is the copper ion that gives laccases their characteristic blue colour and is the site of substrate oxidation. Type 2 and Type 3 copper ions form the laccase trinuclear cluster, the site of the reduction of molecular oxygen and resultant formation of water (Riva, 2006)

Figure 1-19 shows the catalytic cycle of laccases, which includes the redox reactions of the laccase active site copper atoms.

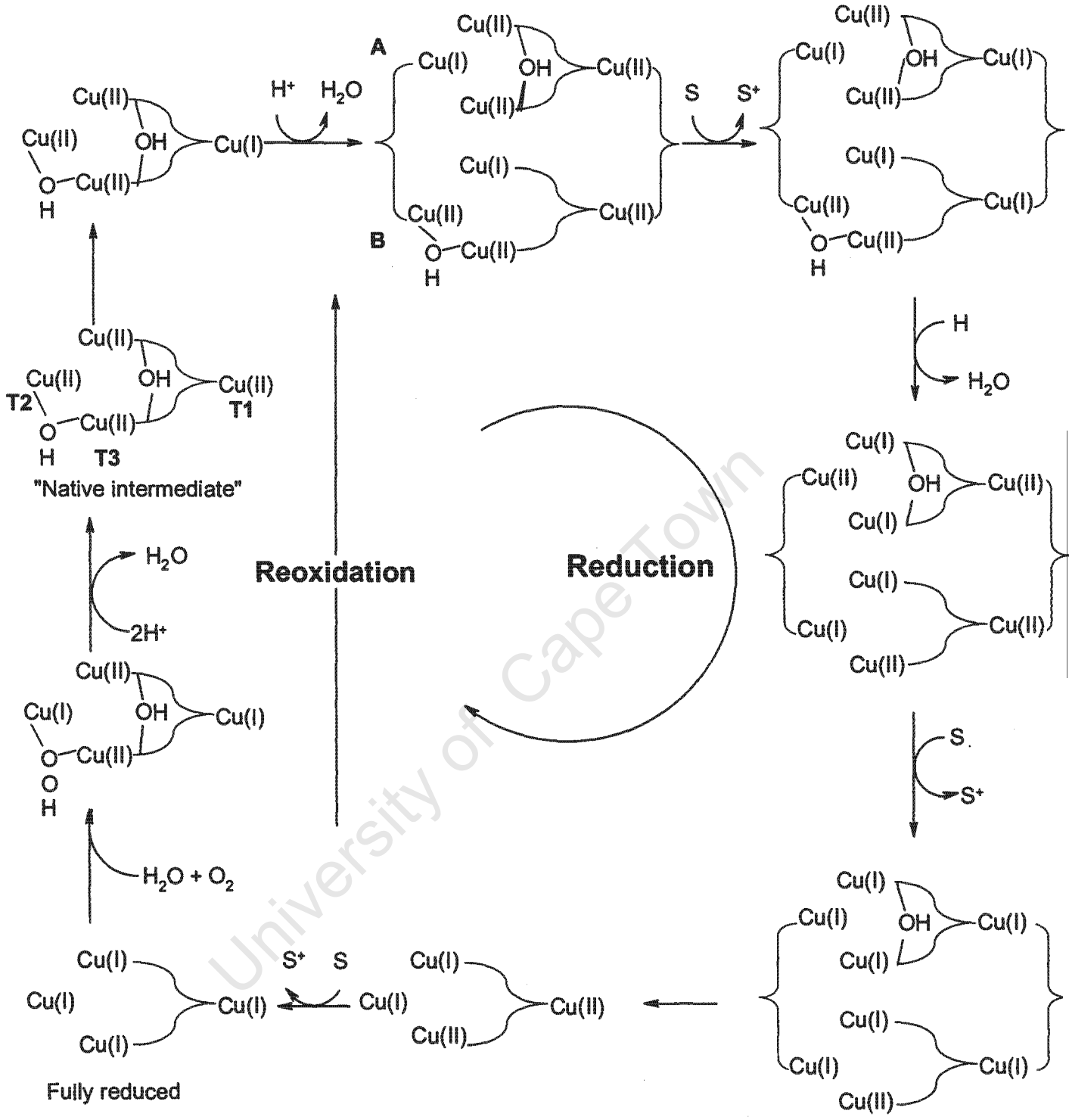


Figure 1-19. Laccase catalytic cycle showing the reoxidation and reduction of copper sites. The substrate reduces the T1 site of the 'native intermediate', and the trinuclear cluster is reduced by the electron. There are 2 possible mechanisms for the reduction of the trinuclear cluster which are shown, A and B. In the former, the T1 and T2 sites together reduce the T3 copper pair, and in the latter, sequential reduction of the coppers in the trinuclear cluster takes place, from the T1 site. The pathway to the fully oxidised form is not shown as it is not catalytically relevant (Solomon *et al.*, 1996).

Multicopper oxidases exhibit either high or low substrate specificity; plant and fungal laccases fall into the latter group and oxidise a wide range of electron-rich substrates such as diphenols, aryl diamines and aminophenols. Their high Km values suggest the absence of a substrate binding pocket and therefore the possibility of outer-sphere oxidation (Xu, 1996).

The laccases can be broadly divided into two main groups: the fungal and plant laccases, although laccase is also present in other organisms. All the plant laccases characterised thus far have a single subunit. A synergy exists between laccases and peroxidase during lignin biosynthesis; plant laccases are able to polymerise monolignols to olignols whilst peroxidase is able to synthesise extended polymeric lignin from oligolignol (Sterjiades *et al.*, 1993). The monolignol precursors in lignin biosynthesis are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol.

1.12.2 Fungal laccases

Laccases have been isolated from various fungal species and isozymes have been detected in many. Most fungal laccases are glycosylated monomers or homodimers except for a few such as the laccase from *Podospora anserina I* which has 4 subunits (shown in Table 1-3) (Solomon *et al.*, 1996).

Table 1-3. Properties of some plant and fungal laccases (adapted from Solomon *et al.*, 1996).

Source	Number of Subunits	Percentage Glycolysation	Molecular weight (kDa)
Plant Laccase			
Rhus vernicifera	1	45	110
Rhus succedanea	1	-	130
Acer pseudoplatanus	1	40-45	97
Pinus taeda	1	22	90

Fungal Laccase			
Trametes versicolor	1	10.12	64.65
Neurospora crassa	1	11-12	64-64.8
Pleutrotus ostreatus	1	12.5	59-64
Trametes villosa 1	2	7	126
Rhizoctonia solani 4	2	10	132
Myceliophthora thermophila	2	14	160
Scytalidium therophilum	1	-	75-80
Polyporus anisoporus	1	-	57.5
Phlebia radiata	1	11.8	64
Podospora anserina I	4	20-23.7	390

1.12.3 Function of fungal laccases in nature

Some of the functions of laccase in fungi are outlined below:

- Pigment formation. Laccases are associated with fungal pigmentation, and laccase-deficient fungal mutants have pigmentation abnormalities. Mutants that produce atypical amounts of laccase isozymes, have been observed to have pigmentation irregularities (Esser *et al.*, 1970; Tanaka *et al.*, 1992). Normally the fungus *Aspergillus nidulans* produces green spores, but the laccase-deficient mutant spores are yellow instead. The mutants revert if grown in media supplemented with partially purified laccase (Clutterbuck, 1972).
- Lignin degradation. The role of laccase in fungi is synergistic with other enzymes such as peroxidase. Some fungi can still degrade lignin although they produce no laccase. However some laccase-deficient mutants cannot degrade lignin, but revert if supplemented with purified laccase in their growth media. *In vitro* studies have shown that laccase can catalyse the degradation of lignin models as well as the repolymerisation of lower molecular weight compounds (Leonowicz *et al.*,

1985). Thus, fungal laccases can polymerise low molecular weight phenolic compounds or depolymerise lignin.

- Detoxification. Lignin degradation results in the formation of potentially toxic compounds such as phenols. It has been proposed that extracellular laccase is produced in response to this and, possibly, laccase-deficient mutants are unable to remove these toxins, leading to their demise. Laccases may also be produced by fungi in order to detoxify phenols released by other organisms in their environment, and this might give them a competitive advantage, for example in soils, where they are commonly found (Solomon *et al.,* 1996).

1.12.4 Uses of fungal laccases in industry

Laccases have been used extensively for industrial applications, for example as bleaching agents, in azide detection, ethanol production in lignocellulose and drug analysis for the differentiation of morphine from codeine (Mayer and Staples, 2002). Most applications of laccases are in the bioremediation of phenolic industrial pollutants (Ryan *et al.,* 2005; Mayer and Staples, 2002). The oxidation of phenol derivatives by laccases results in their polymerisation (Lomoscolo *et al.,* 1999). Polymeric phenols precipitate out of solution, and by simple sedimentation and filtration can be separated easily, detoxifying the effluents (Riva, 2006). The laccases used in industry are usually fungal laccases (mostly from white-rot fungi) due to the ease of their production in large scale. Laccase has also been engineered to improve the rate of electron transfer between the active site and an electrode, to improve the efficiency of laccase electron transfer kinetics (Gelo *et al.,* 1999).

1.12.5 The effect of mediators on laccase reactions

The substrate specificity of laccase can be broadened by using mediators. The presence of mediators allows the oxidation of compounds with higher redox potentials to that of laccases (Burton, 2003; Riva, 2006; Thurston,1994). The mediators themselves are

oxidised by laccase and form highly reactive intermeditates which can react with non-phenolic compounds (Bourbonnais *et al* ., 1997, Figure 1-20). Various mediators of laccase have been identified, including 3-hydroxyanthranilate in the case of *P. cinnabarinus* and 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonate, (ABTS) amongst the artificial ones (Pointing, 2001). Most industrial applications of laccases involve the use of mediators (Riva, 2006).

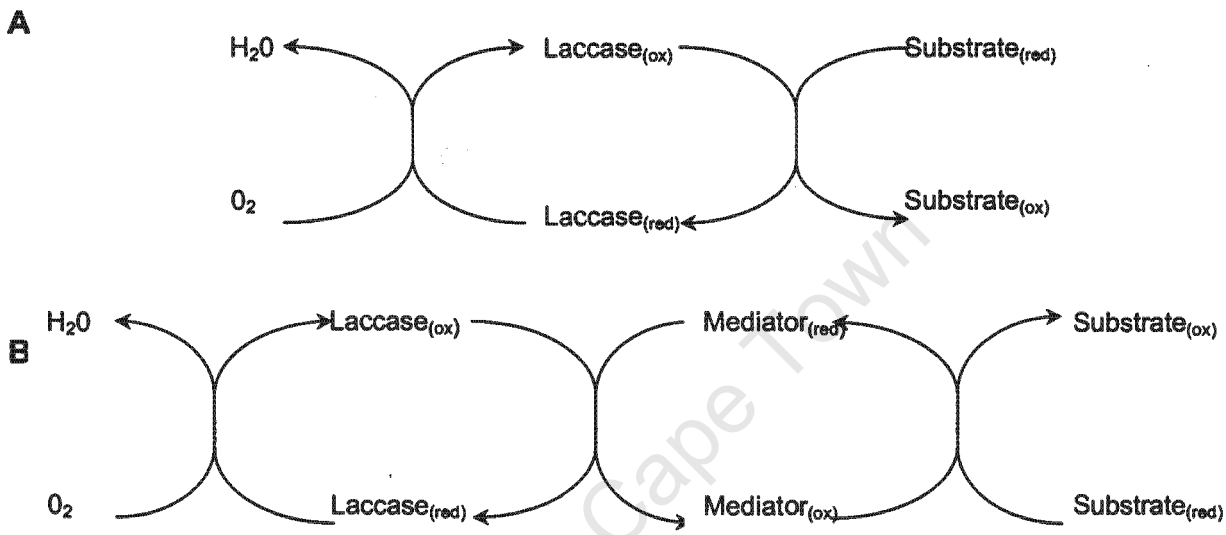


Figure 1-20. Schematic representation of laccase redox cycles in the absence (A) and presence (B) of mediators (Riva, 2006).

1.12.6 Use of laccases in biocatalysis for the industrial production of chemicals

The use of laccases for the synthesis of fine chemicals has not extensively progressed to industrial levels, although several small lab scale reactions have been reported (Burton, 2003). This is due to several factors including the difficulty in controlling the specificity of the polymerisation process as well as the polymerisation of oxidised target products (Kobayashi and Higashimura, 2003). For instance, the production of vanillin from ferulic acid catalysed by *Pyconoporus cinnabarinus* whole cells have been reported, but the

products were polymerised by extracellular laccase in the media (Falconnier *et al.*, 1994). The synthesis of cytotoxic compounds with similar structure to those of actinomycin antibiotics have been reported (Trejo-Harnandez *et al* ., 2001). Using mediators, the oxidation of non-phenolic substituents (benzyl, benzylic, allylic, aliphatic and propagyl alcohols) of lignin-model compounds has been reported (Riva, 2006)(Figure 1-20)

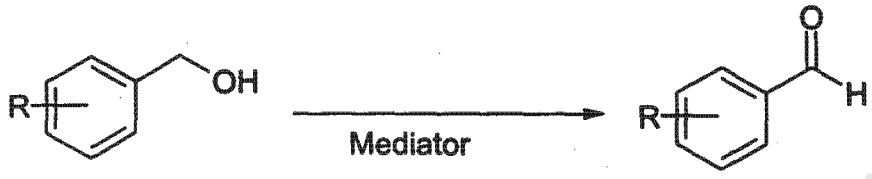


Figure 1-21. The laccase-catalysed oxidation of benzyl alcohols to corresponding aldehydes (Riva, 2006).

1.12.6.1 Laccase reactions with ferulic acid

Ferulic acid reactions with laccase have been studied previously and complex mixtures of products were reported (Carunchio *et al.*, 2001, Mustafa *et al.*, 2005). The initial products from the reaction between ferulic acid and laccase from *Pyricularia oryzae* resulted from the dimerisation of ferulic acid to form (Z)-β-{4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxy cinnamic acid (A in Figure 1-22) and trans-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid (B in Figure 1-22) (Carunchio *et al.*, 2001). Mustafa *et al.*, (2005) investigated the formation of coloured products from the reaction of ferulic acid and laccase, for application as food colouring agents. The laccase that was used was a fungal laccase from *Myceliophthora thermophila* and the reactions were performed in biphasic reaction mixture to improve the stability of the products. Elucidation of the structures of the compounds was not performed (Mustafa *et al* ., 2005). Tranchimand *et al.*, (2006) reported the formation of a compound rac-2 (C in Figure 1-22) from the ferulic acid reaction with laccase from *Trametes versicolor* , *Melanocarpus albomyces* and *Trametes* C30. The difference in products obtained to those obtained by

Carunchio *et al* ., was proposed to be due to different physico-chemical properties of the two reaction media or different enzyme preparations (Tranchimand *et al.*, 2006)

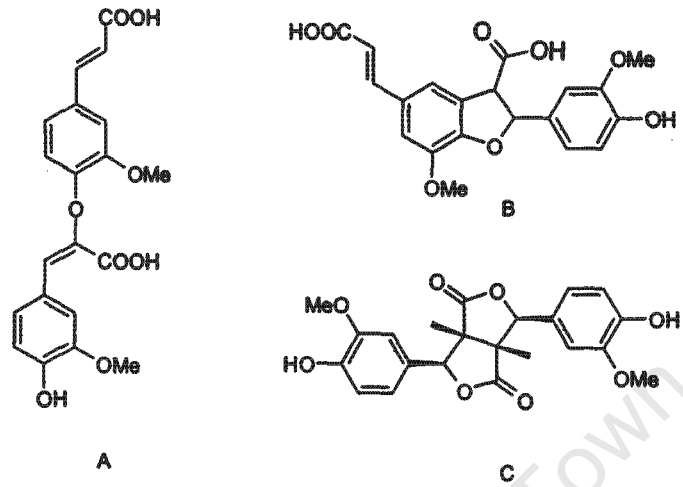


Figure 1-22. Structure of ferulic acid dimers formed from a ferulic acid reaction with laccase. A is (Z)-β{4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxy cinnamic acid (product P1) and B is trans-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid (product P2) (Carunchio *et al.*, 2001). C is Rac-2 reported by Tranchimand *et al.*, (2006)

1.12.7 Laccase reactions in organic solvents

Most substrates of laccase are insoluble in water and hence there is a need to develop laccase reactions in organic solvent. The effect of medium engineering, previously mainly reported for hydrolases, was observed for laccases (Intra *et al.*, 2005). The use of water miscible co-solvents was shown to affect laccase activity in that they were shown to act as enzyme inhibitors. This was correlated to the denaturating effects of the solvents (Rodakiewicz-Nowak *et al.*, 2000). However, the use of laccases in water-immiscible solvents has been successfully reported (Lugaro *et al.*, 1973; Milstein *et al.*, 1989; Mustafa *et al.*, 2005). The laccase reactions discussed in the previous section were performed in biphasic reaction systems with the exception of the Carunchio report, which was performed in aqueous system. Laccase mediated oxidation of estradiol in organic

solvent yielded dimers of the hormone were reported by Nicotra *et al.*, (2004). Milstein *et al.*, (1989) successfully performed the oxidation of aromatic compounds using laccase immobilised on Sepharose beads.

1.13 Project hypothesis and objectives

The scope of the research undertaken for this dissertation was part of a large collaborative initiative between academia (University of Cape Town (U.C.T.), University of the Western Cape (U.W.C.) and University of Fort Hare (U.F.H.), South Africa, industry (Afprod) and the Council for Scientific and Industrial Research, (C.S.I.R). The relationship of the collaboration is illustrated below in Figure 1-23. The maize was supplied by Afprod, a South African company that makes starch products from maize. The company was investigating the synthesis of valuable by-products from waste generated in their operations. Together with the C.S.I.R a copyrighted extraction process for ferulic acid from the maize waste was developed. Research conducted at the University of Cape Town, as described in this thesis, was to involve the synthesis (using different biocatalysts), purification and characterisation of value added antioxidant compounds at laboratory scale.

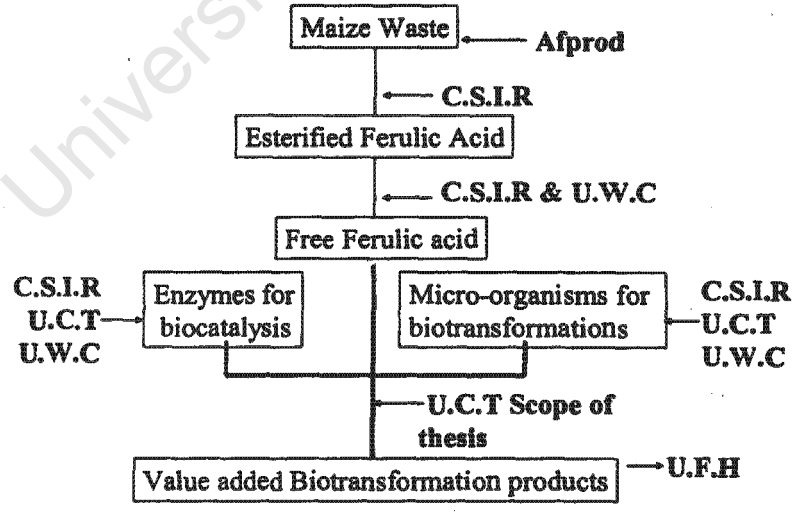


Figure 1-23. Relationship of the collaboration of which the research described in this thesis included. The bold lines indicate the scope of work described in this thesis.

The aim of this study was to develop industrially applicable biocatalytic processes for the synthesis of high value derivatives of ferulic acid. These compounds could have applications in the flavour and/or nutraceutical industry.

Hypothesis: The biocatalytic conversion of ferulic acid can provide biologically active derivatives with higher biological activity than their precursors.

Specific objectives:

1. To assess the current routes for ferulic acid bioconversion and explore novel routes for new ferulic acid biotransformations.
2. To explore the use of laccases as biocatalysts for the synthesis of ferulic acid derivatives. Specifically the synthesis of the flavour compounds vanillin and vanillic acid from ferulic acid using laccase from ferulic acid was to be investigated.
3. To use lipases, which are well characterised biocatalysts, for the synthesis of ferulic acid esters similar to components of γ–oryzanol, a well known compound with potent biological activity related to its antioxidant activity.
4. To conduct an investigation into the biological activity of the biocatalytically synthesised compounds.

2 Bioconversion of ferulic acid using laccase

2.1 Introduction

This chapter describes the production of laccase using an air-lift bioreactor, and its application as a biocatalyst for the bioconversion of ferulic acid in different reaction media. Methods for detection of products were developed and the products were analysed for antioxidant activity. There are some reports of reactions of ferulic acid with laccase in aqueous conditions (Carunchio *et al.*, 2001) and a recent study reported the use of similar conditions to those described in this chapter (Mustafa *et al.*, 2005). However, this thesis describes the characterisation and analysis of these products in organic, aqueous and biphasic media.

The potential application of the fungal enzyme laccase in ferulic acid reactions was investigated in the present study, with the aim of producing the flavour compound vanillin. This was on the basis of preliminary unpublished results by Koteshwar *et al.*, (2000).

2.2 Air lift reactors for the production of laccase

A common misconception concerning the use of enzymes in industrial processes is that they are expensive (Faber, 1995). This is not necessarily the case, as many enzymes can be inexpensively produced by modern molecular and fermentation technology, which provides for high levels of enzyme production, thereby reducing costs. Some easily fermentable fungi can readily produce laccases at very low costs (Ryan *et al.*, 2005). Our research group has optimised the production of laccases from the *Trametes* species using airlift reactors (ALR) for the fermentation. Airlift reactors make good bioreactors as they provide highly oxygenated environments which are often essential for fungal cellular growth. They are also particularly useful for filamentous fungi, due to the well-mixed,

low-shear environments they provide (Klein *et al.*, 2002). The superiority of airlift reactors over other low shear systems such as bubble columns, is attributable to the enhanced solid-liquid mass transfer afforded in air lift reactors. Air-lift reactors designed in our research group were used in this study for the production of laccase from the fungi *T. pubescens* and *T. versicolor.*

2.2.1 Immobilisation of laccase

Many drawbacks encountered in biocatalytic processes are due to the nature of the enzyme catalysts. Because enzymes are proteins, they are sensitive to various factors that are commonly encountered in industrial process such as mechanical shear forces, denaturation by solvents and auto-oxidation which results in loss of activity (Faber, 1995). They are also difficult to recycle due to the difficulty of separation of enzymes from substrates and products. This is an important factor in industrial processes for economic reasons (Katchalski-Katzir and Kraemer, 2000). Furthermore, substrate and product inhibition of enzymes may occur, adding further difficulties to their use in industrial processes. Fortunately, many of these problems may be ameliorated by enzyme immobilisation. Immobilised enzymes were defined as "enzymes physically confined or localised in certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously" by Katchalski-Katzir in 1971 (Katchalski-Katzir and Kraemer, 2000). There are many immobilisation techniques in which either the enzyme is attached or adsorbed onto a solid support, or the enzyme molecules can be cross-linked to each other. Entrapment of enzymes in matrices such as polymers, gels, or in or on membranes, is another immobilisation technique that is widely employed. Immobilisation has greatly increased the mechanical, pH and temperature stability of biocatalysts, allowing recycling and reuse. The selectivity and catalytic activity of the enzymes can be significantly altered, often advantageously (Faber, 1995). An additional technical advantage of immobilisation is that it allows heterogeneous catalysis (Katchalski-Katzir and Kraemer, 2000).

In this study, Eupergit® C was used for the immobilisation of laccase. D'Annibale *et al.,* (2000) previously described the method in 2000, and it was selected as a suitable system for our study because the resulting biocatalyst is highly stable and compatible with common reactor types (Katchalski-Katzier and Kraemer, 2000). This would be necessary for future scale up of the biocatalytic reaction. Eupergit® C consists of macroporous acrylic beads which, predominantly, bind protein via their oxirane groups, which react covalently with the amino groups of the protein. Eupergit® C can also react with protein sulfhydryl and carboxylic groups (Katchalski-Katzir and Kraemer, 2000).

The reactions of fungal laccases with phenolic compounds have been reported to yield polymers which led to the hypothesis that ferulic acid reactions with laccase would yield polymers. The polymerisation of ferulic acid would potentially yield higher antioxidant compounds which would have better applications as antioxidants. It was postulated that, the laccase-ferulic acid reaction would be engineered using medium engineering, to produce the flavour compounds, vanillin and vanillic acid.

The main objective was to produce value added compounds from ferulic acid using a stable biocatalyst from laccases that could be used in future reactions applicable with industrial applications. One very important criterion for enzyme use in industries is their stability (Martin *et al.,* 2003), thus the immobilisation of laccase would be investigated. Immobilisation of laccase on Eupergit® C was to be performed as it had been established as a immobilisation carrier for laccase.

The effect of immobilisation of laccase on the ferulic acid-laccase reaction on the reaction products was to be investigated, as well as the effect of the pH the enzyme was immobilised in, on the reaction products.

An investigation into the nature of the ferulic acid-laccase reaction products formed by different reaction media was to be conducted, as well as an investigation into the effect of the presence of a laccase mediator on the reaction products.

2.3 Materials and Methods

2.3.1 Materials

All materials for the culture media, acetone, ammonium persulphate, glycerol, and ammonium sulphate were obtained from SAARCHEM (South Africa). HPLC grade methanol, acetic acid and dioxane and silica gel plates were obtained from Merck. Ferulic acid and other standard phenolic compounds, DPPH, bovine serum albumin, commercial *T. pubescens* laccase, acrylamide and bis-acrylamide were obtained from Sigma – Aldridge, South Africa. ABTS was purchased from Roche. Eupergit® C was a kind gift from Degussa, Germany.

2.3.2 Laccase production

The method used for laccase production was previously described by Ryan *et al.,* (2005). *T. pubescens* CBS 696.94 and *T. versicolor* PPR1 3845 were grown in 500 mm x 110 mm, 3.5 L draft tube airlift bioreactors (Figure 2-1). 200 mL of *Trametes* Defined Medium (TDM) was inoculated with a homogenised extract of the respective fungus grown on solid medium as a starter culture. The starter cultures were grown for 4 days at 28°C, shaken at 175 rpm. 3.3 L TDM was autoclaved in the bioreactors at 121°C for 10 minutes. The starter cultures were then aseptically added to the bioreactors to start the biomass production. At day 6, 30 mL (0.86 %) autoclaved cresolic gas stripped liquor from a coal gasification plant (Ryan *et al.,* 2005) was added in 50 % TDM, to induce laccase production over 5d. After 14d growth, the enzyme was harvested. The medium was centrifuged at 15 000 rpm for 15 minutes. to remove fungal biomass then freeze-dried or purified. Recipes for all media are in the Appendix section.

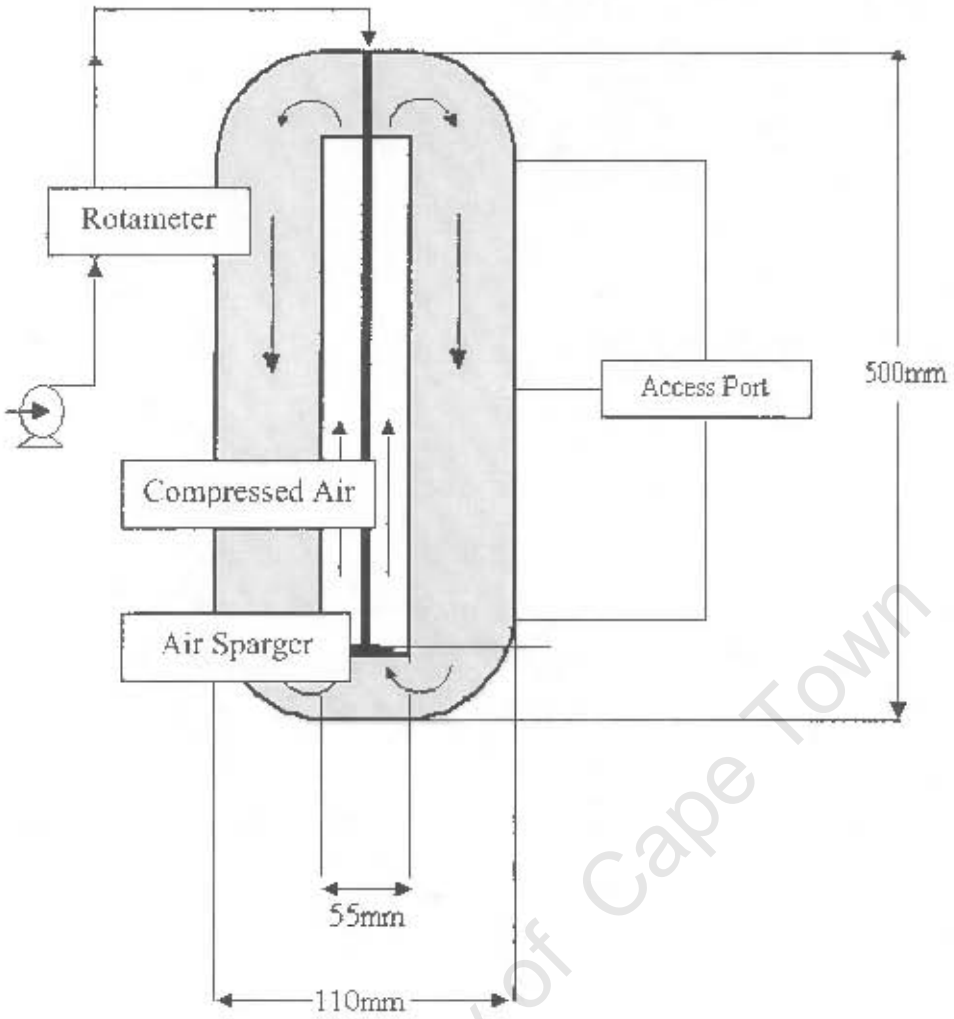


Figure 2-1. Schematic diagram of an air-lift bioreactor used for the growth of fungi in order to harvest the extracellular enzyme, laccase (from Ryan *et al.*, 2005).

2.3.3 Laccase purification

2.3.3.1 Acetone precipitation

To purify the laccase from the fungal growth medium, an equal volume of cold acetone was added slowly to laccase-containing medium, while stirring gently. The mixture was allowed to stand for 1h and centrifuged at 17,675 x g (10 000 rpm in a JA-10 Beckman rotor) for 10 minutes. The pellet was washed with distilled water, and resuspended in 0.1x to 0.2x the initial volume, in 0.1M sodium acetate buffer, pH 5.

2.3.3.2 Successive acetone precipitation of laccase from medium

Medium containing laccase produced in the air lift reactors with *T. pubescens* was treated with increasing proportions of cold acetone. Cold 10, 30, 50 or 70 % v/v acetone was added slowly to the laccase containing growth medium and gently stirred. The mixture in each case was left for 30 minutes on ice and then centrifuged at 17,675 x g (10 000 rpm in a JA-10 Beckman rotor) for 10 minutes.

2.3.3.3 Ammonium sulphate precipitation of protein from medium

Medium containing laccase produced in the air lift reactors from *T. pubescens* was treated using ammonium sulphate. Successive amounts of ammonium sulphate (to reach 10, 30, 50, 70 and 90 % saturation) where added in order to precipitate the protein. The procedure was performed on ice and the pellets obtained by centrifugation at 17,675 x g (10 000 rpm in a JA-10 Beckman rotor) for 15 minutes. The pellets were resuspended in 0.1 M sodium acetate buffer (pH 5) and the collected protein was dialysed against water at 4°C for 18h.

2.3.4 Protein quantification of enzyme samples

The Bradford assay (Bradford, 1976) was used for the determination of protein concentration. 0.1 mL of protein sample or standard was reacted with 2.9 mL of Bradford's reagent. The mixture was mixed gently by inversion and was incubated at room temperature for 5 minutes. The optical density of the solutions was determined at 595 nm. Solutions of between 0.1 and 1.5 mg/mL bovine serum albumin (BSA)in phosphate-buffered saline were used as standards. The standard curve used to quantitate the protein is shown below in Figure 2-2 and the composition of Bradford's reagent is given in the Appendix.

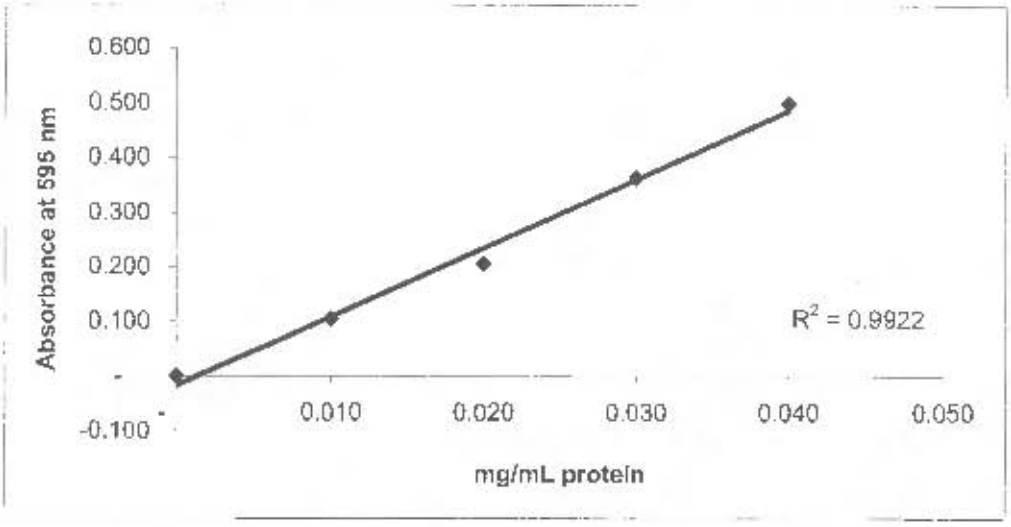


Figure 2-2. Standard curve for protein determination. R^2= 0.992

2.3.5 SDS-PAGE analysis

10 % vertical polyacrylamide gel electrophoresis in 0.1 % SDS was performed to separate and visualise purified protein, as described by Laemmli (1970). 20-50 µg protein samples were boiled for 5 minutes in dissociation buffer and run at 100 to120V for an hour at room temperature. Coomassie staining solution (See appendix) was used to visualise the protein bands on the gel. Rainbow markers (Sigma) or low range molecular weight markers (Roche) were also run in order to confirm the molecular weight. Commercial *T. versicolor* laccase was run as a reference.

2.3.6 Laccase assay

Laccase activity was determined spectrophotometrically based on its oxidation of the chromophore 2-2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid, ABTS) (Wolfenden and Willson, 1982). Oxidation of the ABTS was determined by measuring the increase in absorbance of an assay mixture at 420 nm (ε=36 000 M^{-1}). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute.

0.17 mL of sample was added to 0.5 mM ABTS in 3 mL 0.1 M sodium acetate buffer pH 5 (Roy-Arcand and Archibald, 1991). All measurements were carried out using an α-Helios Helicam spectrophotometer, at room temperature.

2.3.6.1 Concentration of purified enzyme

A concentrated enzyme solution was prepared, by removing excess water from the solution by absorption with polyethylene glycol, PEG. Snake skin dialysis tubing (Pierce) with a molecular weight cut off of 3 500 Da was soaked in distilled water overnight. 25 mL of dilute purified enzyme solution was placed in it and tied securely with string and placed in 20 % w/v PEG solution. The dialysis was performed overnight at 4°C until approximately 1 mL of solution was left. The enzyme activity and protein concentration of the enzyme was determined using the methods described previously.

2.3.6.2 Enzyme immobilisation

The method used was a modification of that described by D'Annibale *et al.* (2000). 50 mg Eupergit® C was mixed with 308 U (10 mg) laccase enzyme, and binding buffer (potassium phosphate buffer, 1.5 M at pH 7 and pH 5.7, or sodium acetate buffer at pH 5). The polymer was placed in a 1.5 mL Eppendorf tube with 500 µL of concentrated enzyme. 0.5 mL binding buffer was added and the mixture was vortexed for 2 min. The mixture was then put on a rocker at 25°C for 24h. The beads were washed sequentially with 1 mL de-ionised water and 1 M NaCl. The washes were retained and assayed for any residual enzyme activity. The washed beads were stored at 4°C in binding buffer. Some beads were lyophilised and stored at 4°C. More enzyme was immobilised using a scale-up of the procedure described to obtain more immobilised biocatalyst.

2.3.7 Biocatalytic reactions with laccase

2.3.7.1 Aqueous reactions

Non-immobilised laccase (1, 10 or 20 U) was added to sodium acetate buffer (pH 5, 0.1 M; 40 mL) containing ferulic acid (10 mM). In most cases, organic solvent (THF, MeOH or acetone) was included, making up 2.5 % v/v of the buffer volume. Reactions were run overnight (19h) or for 48h. When a mediator was included, 0.5 mM ABTS was added to the reaction. Reaction mixtures were extracted with ethyl acetate and extracts were analysed by high performance liquid chromatography, HPLC, liquid chromatography-mass spectroscopy, LC-MS, and thin layer chromatography, TLC in some cases (see below for methods).

2.3.7.2 Reactions in organic media

The effect of different media on the reaction products was assessed. 10 mM ferulic acid (10 mL) was reacted with 100 mg *T. pubescens* laccase immobilised in different buffers (at pH 5, 5.7 and 7), in 100 % ethyl acetate, a biphasic system comprising equal amounts ethyl acetate and 0.1 M sodium acetate buffer (pH 5.7) or in sodium acetate buffer only, all at 40°C.

2.3.8 Analysis of products

2.3.8.1 High Performance Liquid chromatography (HPLC)

A LaChrom L-7400 (Merck, Hitachi, Germany) HPLC system was used for all HPLC analysis. This was equipped with a UV/VIS detector, an 80 sample autosampler and a 20 µL auto injector. Optimisation of different mobile phases was performed, including gradient elution of acetonitrile and acidified water (pH 3), and various ratios of methanol, water and acetic acid (68:40:2 and 60:40:2 water: methanol: acetic acid, v/v). A 5 µm C18 Waters (250 mm X 4.6 mm) reverse phase column and guard column were used. 10 µL samples were run with a flow rate of 1 mL/min and detected at 280 or 300 nm. 10

mM pure phenolic standard compounds were dissolved in methanol and analysed to confirm the identity of the peaks in the reaction mixtures by comparison.

2.3.8.2 Thin Layer Chromatography (TLC)

TLC of the products of biocatalytic conversion of 10 mM ferulic acid/laccase reaction was conducted using aluminium-backed silica gel 60 F_{245} (Merck) plates with benzene/dioxane/acetic acid (90:25:4; v/v) as the mobile phase. The compounds were visualised by exposure to UV light and/or elemental iodine.

2.3.8.3 Liquid chromatography - mass spectroscopy (LC-MS)

Full scan LC-ESMS was conducted in the negative mode scanning between 0-1000 or 0-700 m/z. Initially, 25 % acetonitrile was used as a mobile phase, and subsequently 35 % acetonitrile/0.025 % formic acid in water. The column used was Luna C18; 3µ; 2X150 mm. The flow rate was 100 µL/min, and 5µL samples were injected. A voltage of 3.0 kV was applied to the capillary tube. The temperature of the source was kept at 70°C. Ions were generated using nitrogen and nebulising gases at flowrates of 250 L/hr and 13 mL/min. Voltage was set at 25V.

2.3.8.4 Analysis using gas chromatography

A Thermofinigan Focus GC with an ALPHA DEX™ 120 capillary column (30 m x 0.25 mm x 0.25 μm film) was used under the following conditions:

Initial temperature	135°C
Column hold time	10 min.
Final column temperature	220°C
Ramp Rate	10°C/min.
Injector temperature	280°C
Detector temperature	300°C
Total run time	40 min.

2.3.8.5 Gas Chromatography-Mass Spectroscopy, GC-MS

The GC-MS instrument used was an Agilent 6890 N GC-MS using helium as the carrier gas. The MS was conducted in the negative mode scanning from 50-450 mz. The column used was an HP5 MS column (30 m x 250 μm x 0,25 μm). The method followed was as below:

Initial temperature	60 °C
Column hold time	1 min.
Final column temperature	300°C
Ramp rate	25°C/min
Final column hold time	5 min.
Injector temperature	250°C
Total run time	15.6 min.

2.4 Results and Discussion

2.4.1 Production of laccase and recovery from growth medium

In order to obtain laccase enzyme to use as a biocatalyst, a concentric draught-tube internal loop airlift bioreactor was used to grow the fungi *T. pubescens* and *T. versicolor*. These fungi were chosen as they are good laccase producers and are used routinely by our research group. Laccase is an extracellular enzyme and was secreted into the medium by the fungi used. It has been found that laccase production can be induced by the addition of several chemicals, including, but not exclusively, lignin-like compounds (Gianfreda *et al.*, 1999). A cresolic effluent was previously found to have a prolonged inductive effect on laccase production in *Trametes* spp. (Ryan *et al.*, 2005) and for convenience this method was used in this study to induce laccase production. The inducer was added on day 6 and the laccase activities of the bioreactors were assayed over time. The results are shown in Figure 2-3 below. After 13 days *T. versicolor* had produced higher laccase activity (4.9 U/mL) than *T. pubescens* (1.5 U/mL). Both laccases were kept for further use.

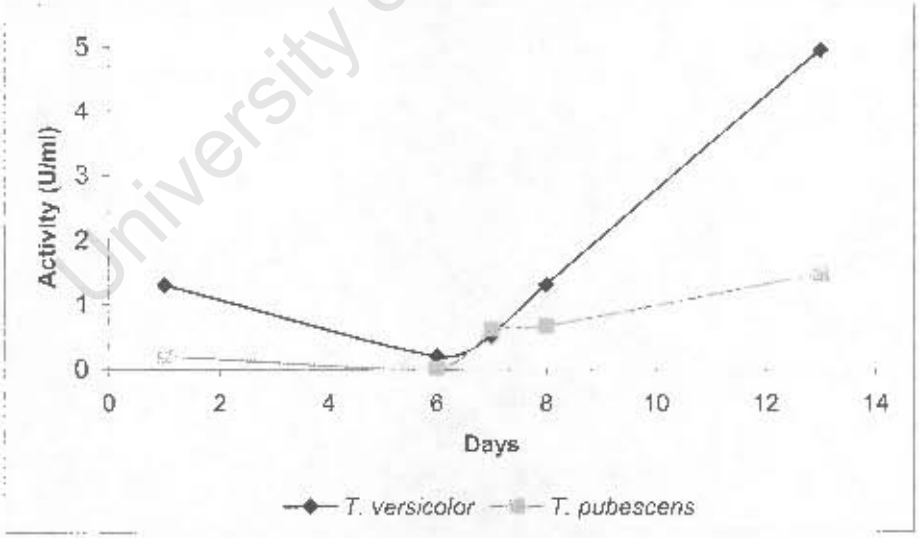


Figure 2-3. Laccase production from *Trametes* species in an air lift bioreactor.

2.4.2 Laccase enrichment from growth medium

Laccase was purified in order to separate the enzyme from the medium so as to use it without interference from medium components and to separate it from other proteins. Precipitation steps in enzyme purification procedures are used to separate a desired enzyme from solution. The solution is then centrifuged to form a protein pellet, which can then be resuspended in a buffer. Proteins differentially precipitate out of solution at different concentrations of precipitating agent (according to their nature). This allows the isolation of a desired protein from inactive protein by using different amounts of precipitating agents. Successive ammonium sulphate and acetone protein precipitation procedures were investigated, but redissolving the precipitated proteins in solution became increasingly difficult for the fractions containing laccase. Precipitation of laccase from the supernatant using 50 % acetone was found to be adequate for enrichment, as shown by the single bands of the laccase mono-subunits obtained by SDS-PAGE, shown in Figure 2-4. The absence of contaminating protein bands was taken to indicate that the protein samples were sufficiently pure for use in biocatalysis reactions.

Figure 2-4. SDS-PAGE showing bands from 50 % acetone precipitated protein. Rainbow molecular weight markers (M), commercial (C)and purified (P) fractions are shown.

A specific activity of 278 U/mg was obtained for the laccase after purification, compared to 12.06 U/mg obtained for the crude extract (Table 2-1).

Table 2-1. Protein purification table for laccase isolation from fermentation medium

Fraction	Volume (mL)	Protein (mg/mL)	Activity (U/mL)	Specific activity (U/mg)	Total activity (U)	Total protein (mg)	Yield %	Fold purification
Crude Extract	200	0.83	10	12.06	2000	2411.6	100	1
Purified extract sup.	400	0.043	0.3	7.14	122.8	17.2	6.1	1.7
Purified extract pellet	200	0.048	10	278.7	2000	9.6	100	23.1

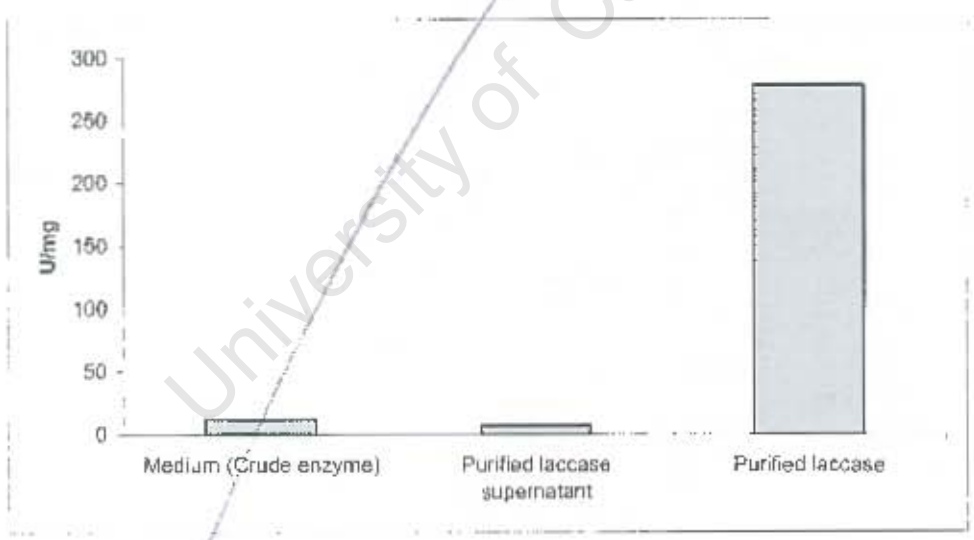


Figure 2-5. Specific activities of laccase samples used for the SDS-PAGE analysis and immobilisation of laccase on Eupergit® C.

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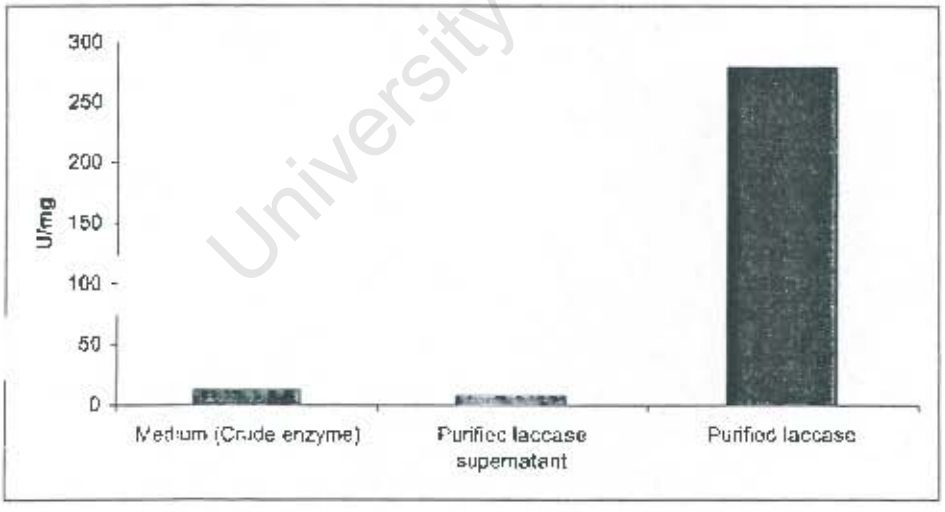


Figure 2-5. Specific activities of laccase samples used for the SDS-PAGE analysis and immobilisation of laccase on Eupergit® C.

2.4.3 Immobilisation of laccase on Eupergit® C.

The method of immobilisation used was as described by D'Annibale *et al.* (2002). The immobilisation of laccase on Eupergit® C was successful with loss of less than 15 % of the initial total enzyme activity left unbound. 98 % (263.4 U) of the laccase was bound on 50 mg solid support. The residual unbound enzyme was removed during the washes and the enzyme activity lost per wash is shown in Figure 2-6 (some enzyme was left unbound before the washes (unbound enzyme in Figure and some was removed during the washes). The assessment of the activity of the immobilised catalyst was problematic because the ABTS-laccase reaction product appeared to be localised on the beads and the reaction products did not diffuse well into the solution, thus making the calculation of the activity of the bound catalyst inaccurate. Diffusional limitations due to immobilisation resulted in very low activities being obtained for the immobilised biocatalyst and have been observed in other studies. These necessitated the modification of assay methodologies (Davis and Burns, 1992; Wilson *et al*., 1994, and Ncanana S.[1]). As different assay methodologies were not possible during the course of this study, the residual activity lost was calculated and an amount of immobilised enzyme used was stated in each case as others have done (Barrato *et al.*, 2006). For the subsequent reactions, 50 % loss of laccase activity during immobilisation was assumed, as D'Annibale *et al* ., (2000) observed a 56 % loss of laccase activity in their study on the immobilisation of laccase on Eupergit® C, the study on which this work was based.

[1] Personal communication

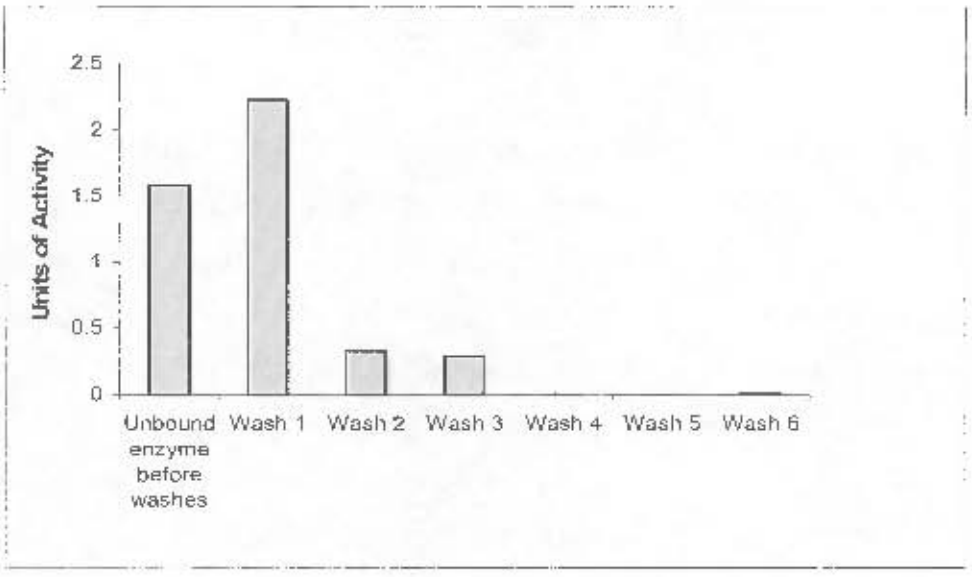


Figure 2-6. Laccase activity during immobilisation of laccase on Eupergit© C in washes.

2.4.4 Reactions of ferulic acid catalysed by non-immobilised laccase in aqueous medium

This section describes an investigation of the ferulic acid reaction catalysed by laccase in aqueous medium. Reactions of non-immobilised laccase with ferulic acid in buffer, at the optimal pH for laccase were performed. Ferulic acid dimers were previously reported as the initial products of this reaction by Carunchio *et al.*, (2001). Mustafa *et al.* (2005), reported the formation of coloured products from the reaction, and recently Tranchimand *et al.* (2006) reported the synthesis of a lignol from ferulic acid (Figure 1-22). The present study was conducted to investigate and characterise the ferulic acid-*T. pubescens* laccase reaction products over time, and to observe the effects of different media on the reaction products.

The reactions between ferulic acid and laccase were conducted at various enzyme to substrate ratios, in order to determine a suitable ferulic acid concentration to enzyme activity ratio, in which reactions were complete within 24 hours. This was done in order to facilitate sampling to assess formation of products over time, without extending the reaction time beyond 24 hours. A ratio of 1 U of laccase activity to 1 mM ferulic acid

was found to yield the best results in a controllable manner, in reasonable time. At this ratio, the detection of intermediate products was possible, as the reactions did not proceed too fast. When only the end products of the reaction were desired for analysis, twice the enzyme activity was used in order, to decrease the time of reaction.

Addition of small amounts of acetone (up to 36 %) or methanol (2.5 %) for the purposes of achieving solution of the ferulic acid did not decrease the conversions, but the addition of THF resulted in inhibition of the reaction. Of the three solvents (methanol, acetone and THF) assessed for solubilisation of ferulic acid for the reaction, methanol was subsequently used as the solvent of choice as the least amount was required. Not only was THF found to inhibit laccase activity, but it also led to the formation of a cloudy white precipitate in the reaction mixture.

2.4.4.1 TLC analysis of the reaction of ferulic acid with non-immobilised laccase products formed in aqueous medium

In order to analyse the products of the reactions, 10 mM ferulic acid (0.25 mmoles) was reacted with 20 U non-immobilised laccase initially. Thin layer chromatography was used as a preliminary tool to assess the progress of the reactions (Figure 2-7). Two products were visible under UV light and a smear, presumed to be due to polymers (with low mobility), were observed.

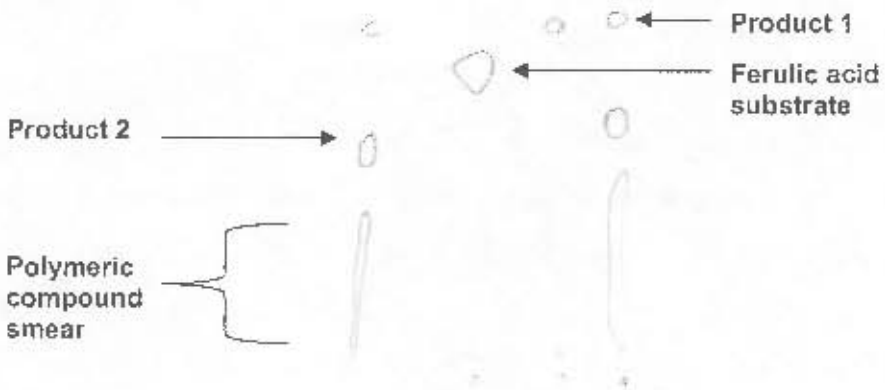


Figure 2-7. Thin layer chromatogram of the products of biocatalytic conversion of 10 mM ferulic acid (0.25 mmoles) by 20 U laccase.

Lane 1: Separation of the reaction mixture.

Lane 2: Ferulic acid showing a different Rf value from the products.

Lane 3: Separation of the aqueous layer of an ethyl acetate extraction of the reaction.

Lane 4: Separation of the organic phase of an ethyl acetate extraction showing two product peaks.

2.4.4.2 HPLC analysis of the reaction of ferulic acid with non-immobilised laccase in aqueous medium

HPLC analysis was performed in order to separate and identify the various reaction products of ferulic acid with non-immobilised laccase in aqueous medium. The reactions resulted in 100 % conversion of the ferulic acid when the ratio of ferulic acid concentration to laccase activity units was 1 mM:1 U within 24 hours (section 2.4.4).

The 10 mM ferulic acid (0.25 mmoles) reaction with 20 U non-immobilised laccase reaction products was sampled periodically and analysed by TLC to obtain the product formation profile. The reaction was found to have reached 100 % ferulic acid conversion

after 18h of reaction time. Separation of components in the reaction product mixture by HPLC is shown in Figure 2-8 below. Table 2-2 shows the approximate retention times of the products formed.

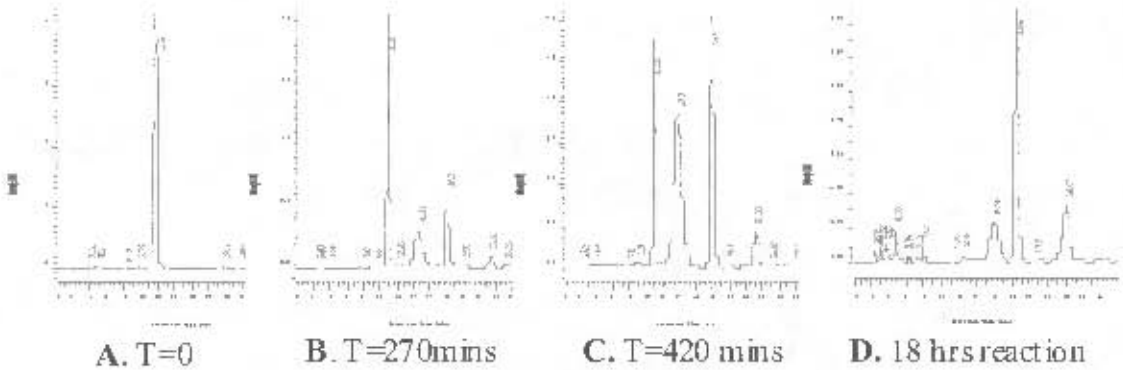


Figure 2-8. HPLC chromatograms showing reaction course for the reaction of 10 mM ferulic acid (0.25 mmoles) with 20 U of purified laccase sampled after 0, 270, 420 min. and 18h. (Mobile phase: methanol/water/acetic acid (30:68:2 v/v); detection at 280 nm).

A.: Chromatogram obtained before the reaction showing the ferulic acid peak.

B.: Decrease in the intensity of the ferulic acid peak and formation of three product peaks after 4.5h reaction time.

C.: Further decrease in the ferulic acid peak and increase in the product peaks after 7h

D.: No ferulic acid peak was detected after 18h showing complete conversion.

Table 2-2. HPLC retention times of the products formed for the reaction of 10 mM ferulic acid (0.25 mmoles) with 20 U of purified laccase, with mobile phase methanol/water/acetic acid (30:68:2 v/v) and detection at 280 nm.

	Retention time
Substrate ferulic acid	11.79 min.
Product 1	15.98 min.
Product 2	18.47 min.
Product 3	24.07 min.

2.4.4.3 HPLC analysis of standard compounds

In order to identify the reaction product peaks, several standard phenolic compounds were analysed by HPLC, and the retention times of the different compounds were determined (Table 2-3). The standards chosen were either phenolic compounds or possible reaction products. The products were found to have retention times different from the standard compounds (Table 2-2) indicating different identities, and hence, mass spectrometry was used to further characterise the products. These products were found to be more non-polar compared to ferulic acid as their retention times were more than those of ferulic acid. The target compounds vanillin and vanillic acid, were demonstrated to be more polar than ferulic acid (lower retention times, Tables 2-2 and 2-3).

Table 2-3. HPLC retention times (minutes) of standard phenolic compounds in different mobile phases

Mobile phase	80:20:2.5 methanol:water:acetic acid v/v	68:30:2 methanol:water:acetic acid v/v
Benzaldehyde		12.63
Catechol	5.78	4.65
Phenol	9.55	6.55
3-Methylcatechol	10.69	
4-Methoxyphenol	11.01	6.97
p-Cresol	19.1	11.07
Protocatechuic acid	5.85	4.32
Gallic acid	4.16	3.47
m- Coumaric acid	22.07	11.65
Ferulic acid	30.29	13.31
Vanillin	16.84	8.89
Vanillic acid	11.75	
Caffeic acid	12.81	7.43
Syringic acid	15.6	
Phthalic acid	7.23	
p-Hydroxyphenylacetic acid	5.26	4.88
p- Hydroxybenzoic acid	8.88	5.91

3,4-Dihydroxy phenylacetic acid	5.23	3.96
1,2,4,5-Benzene tetracarboxylic acid	3.16	2.84
Salicin	5.02	
Isoeugenol		8.5

2.4.4.4 LC-MS analysis of the reaction of ferulic acid with non-immobilised laccase in aqueous medium

The identities of the products could not be confirmed using HPLC and TLC as they had different retention times or R_f values to the standards tested. From the HPLC analysis, it was observed that the compounds were less polar than ferulic acid, the desired products vanillin and vanillic acid, and therefore, the identities of the products were investigated by LC-MS.

Laccase is known to polymerise phenolic compounds and dimeric compounds have previously been reported to be formed as the initial products under some conditions (Carunchio *et al.*, 2001). Ferulic acid dimers were also observed as intermediate and end products of the laccase catalysed ferulic acid reactions in aqueous medium (Lundquist and Kristersson, 1985). The oxidation of phenols by laccase generates quinonoid intermediates. The quinones are very reactive species and can undergo spontaneous non-enzymatic polymerisations (Lundquist and Kristersson, 1985).

The figure below (Figure 2-9) shows the LC chromatogram of the ferulic acid reactions with free laccase in aqueous media and the mass spectra of the resultant product peaks. Three main product peaks were identified. The MS results showed that the two main products, **20** and **22**, had a molecular weight of 771 (Figures 2-10 and 2-16) which indicated the presence of two different ferulic acid tetramers, one with a retention time of 9.71 minutes and the other 17.88 minutes. A lower concentration product, **21**, was also observed with a retention time of 11.87 minutes.

Several free radicals from ferulic acid are possible, depending on the position of the unpaired electron and hence different permutations and combinations can result in different polymers. Several different regio-isomers of ferulic acid dimers and polymers produced by the ferulic acid catalysed by laccase catalysed were indicated by our LC-MS results. The presence of several ions with the same molecular weights, but different retention times, indicated the presence of several isomers.

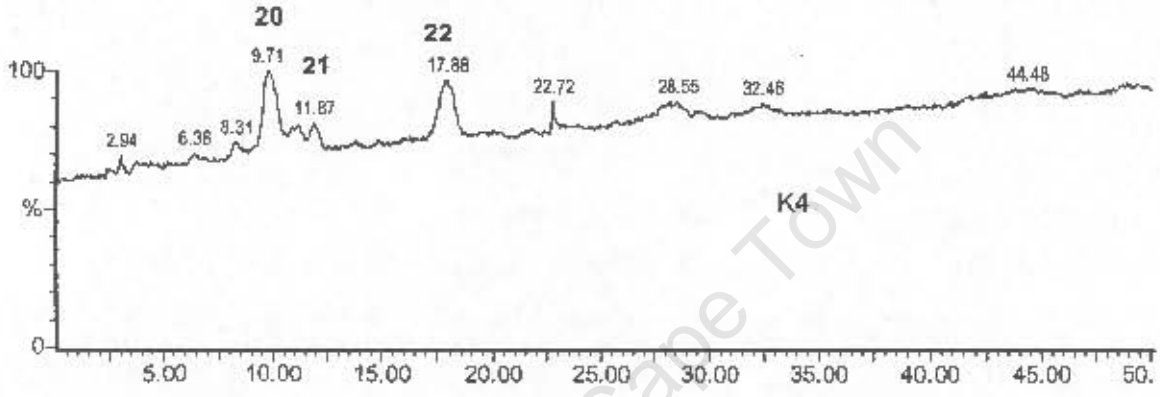


Figure 2-9. Total ion chromatogram (run over 60 min.) of an 18h (25 mL) aqueous reaction of 10 mM (0.25 mmoles) ferulic acid with 20 U of laccase. Three main peaks (20, 21 and 22) were identified.

2.4.4.5 Analysis of product 20

The proposed structure of the tetrameric product **20** is shown below (Figure 2-11). It is proposed that the product is a dimer of the dimer P2 reported by Carunchio *et al.*, (2001) (Figure 2-11a). The fragmentation patterns of product **20** and P2 are almost identical (Table 2-4, Figure 2-12 and Figure 2-10), with the exception of the abundances of the fragments, which varies depending on the ionisation of the sample, and this indicates their identity.

The proposed fragmentation of product **20** which results in fragments with molecular weights similar to P2 is shown in Figure 2-13. The name of the tetramer (found using Chemdraw) is 3-carboxy-2'-(3-carboxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-

dihydrobenzofuran-5-yl)-(2-carboxyvinyl)-7,7'-dimethoxy-2,2',3,3'-tetrahydro-2,5'-bibenzofuran-3'-carboxylate. This is the first report of this compound's synthesis to the best of the author's knowledge (confirmed by searching in SCIFINDER chemical database and the literature).

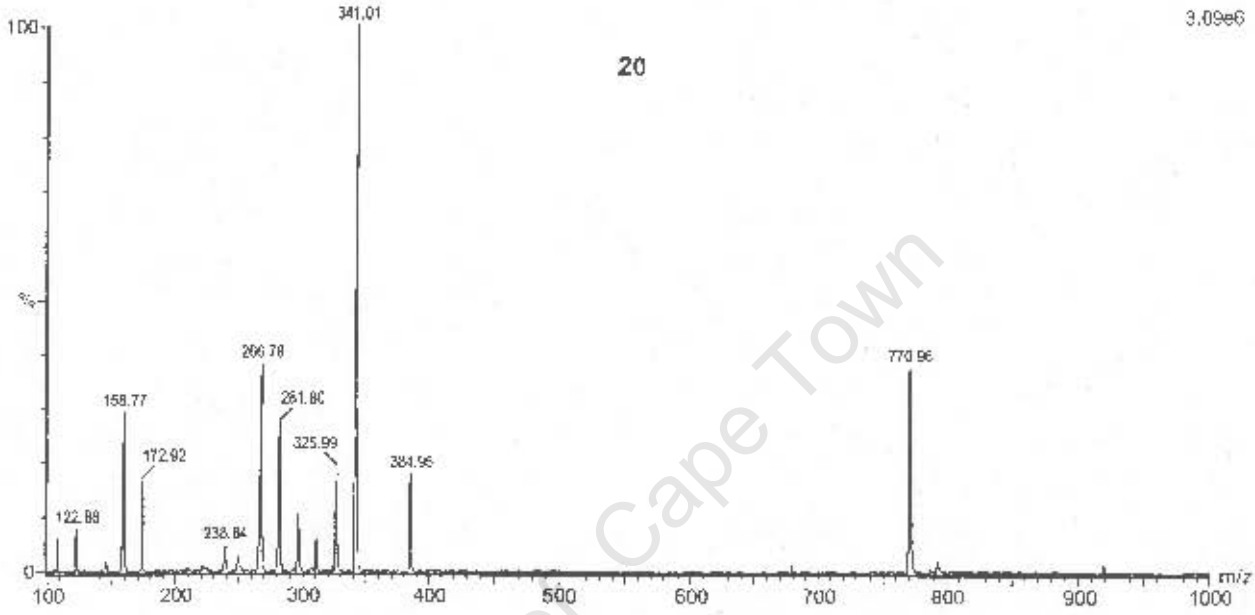
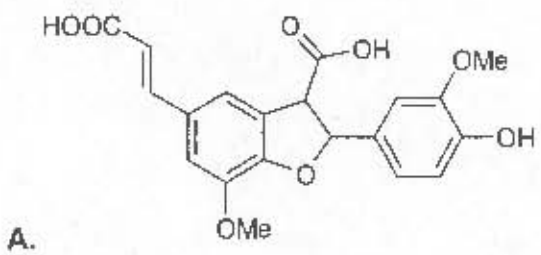
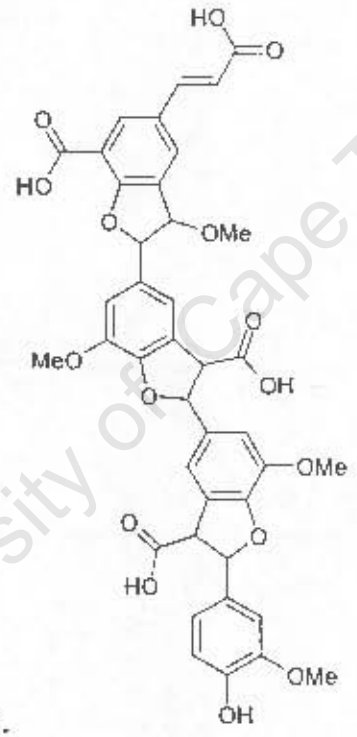


Figure 2-10. Mass spectrum of product 20. Product 20, with a retention time of 9.41 min. was subjected to mass spectrometry. The molecular ion was found to be 771 indicating a tetramer of ferulic acid.



A.



B.

Figure 2-11. Structure of Carunchio's product P2 (A), and (B), product 20, 3-carboxy-2'-(3-carboxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-5-yl)-(2-carboxyvinyl)-7,7'-dimethoxy-2,2',3,3'-tetrahydro-2,5'-bibenzofuran-3'-carboxylate, m/z =771

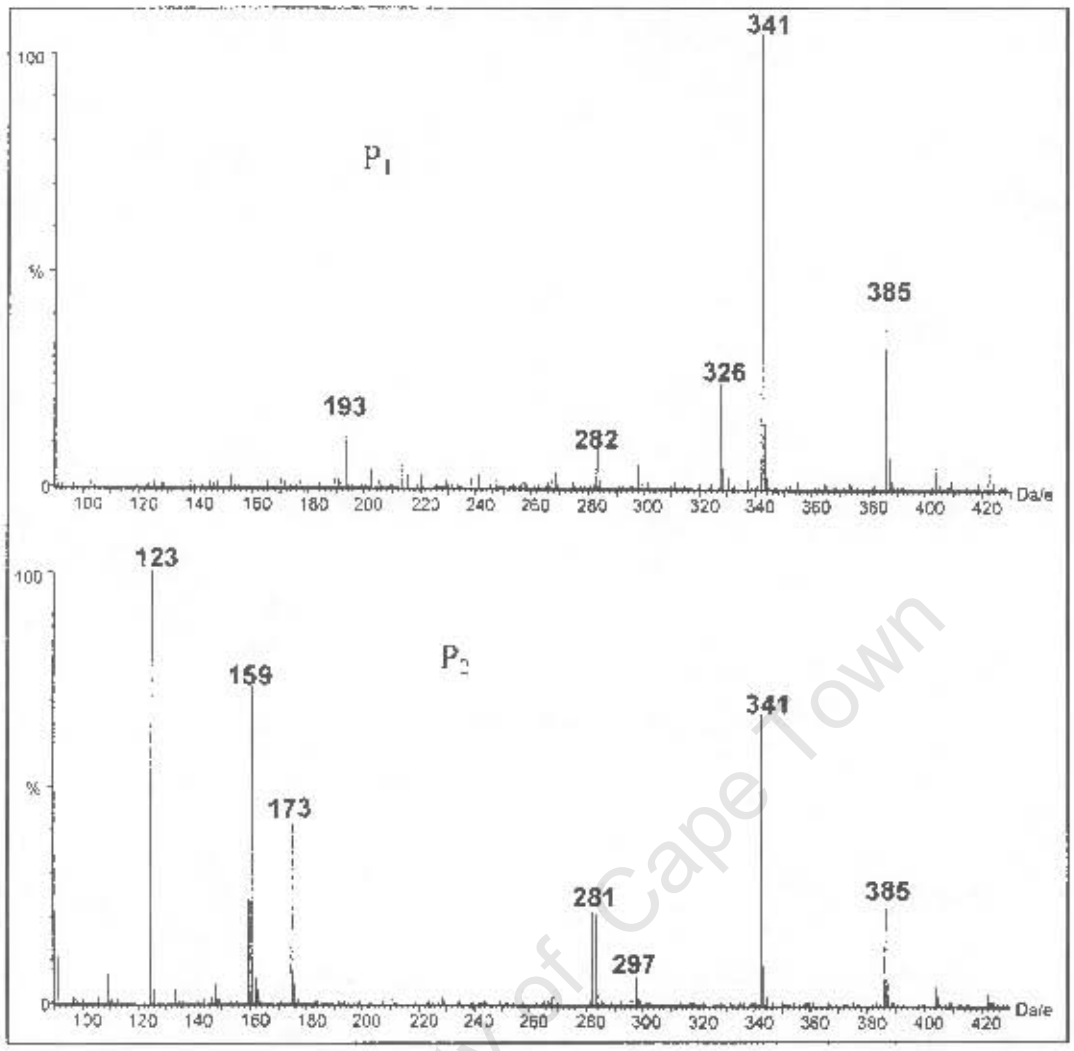


Figure 2-12. Decomposition of the products of a ferulic acid reaction with laccase after 30 min. reaction time, reported by Carunchio *et al.*, 2001. (This figure is taken from Carunchio *et al.*, 2001).

Table 2-4. Mass spectra peaks comparing product 20 with P2 of Carunchio *et al.*, (2001).

Fragment size (m/z)	P2 (Carunchio *et al.*, 2001)	Product 20
123	Present	Present
159	Present	Present
173	Present	Present
281	Present	Present
297	Present	Present
341	Present	Present
385	Present	Present

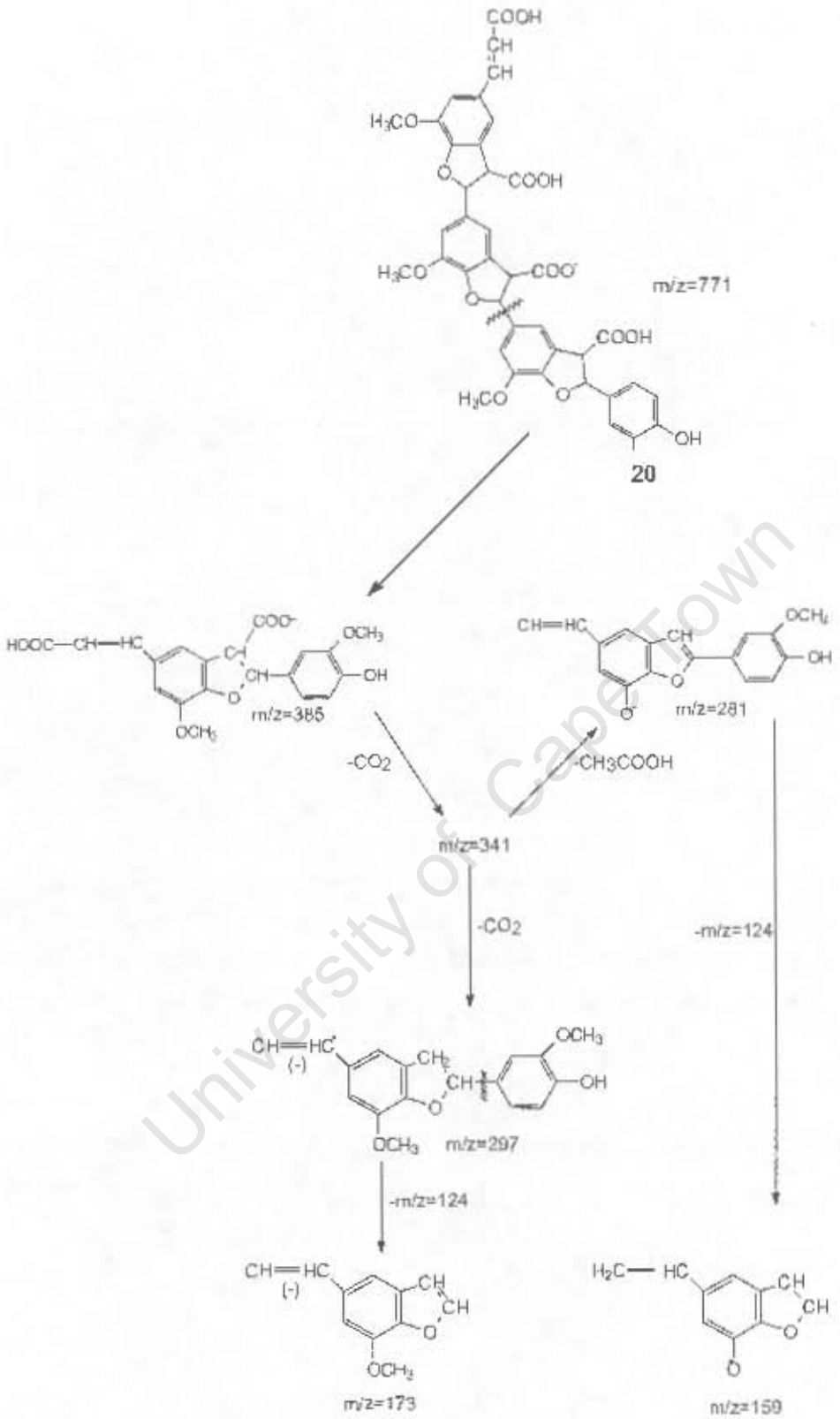


Figure 2-13. Proposed fragmentation pattern of product 20 based on Carunchio *et al.* (2001).

The catalytic ability of laccase relies on its ability to form free radicals. There are three possible intermediate radicals that can be formed from ferulic acid, shown in Figure 2-15 below, which explains and shows the mechanism of polymerisation. These radicals are designated M_O, M_5 and M_β and as a result there are 6 possible dimeric combinations namely β–β, β–5, β–*O*, 5–5 5–*O* and *O*-*O* (Figure 2-15, the numbers designating the position of the free electron) (Carunchio *et al.*, 2001). From each 6 possible combinations, 6 other are possible in the formation of the tetramer, giving a total of 21 possible tetrameric products.

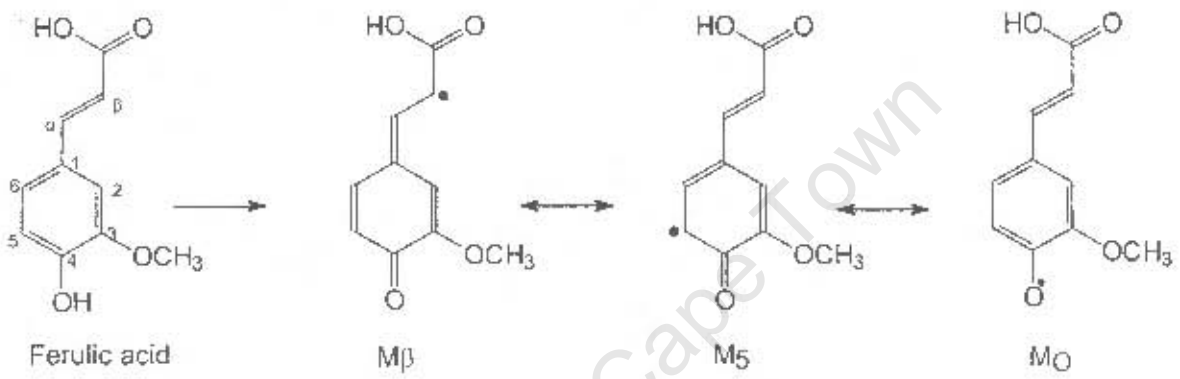


Figure 2-14. Formation of ferulic acid radicals, M_O, M_β and M_5 (Adapted from Carunchio *et al.*, 2001 and Ralph *et al.*, 1992).

The dimerisation of M_β and M_5 forms the monomer of Product **20**. This β–5 linkage of ferulic acid results in the formation of 2 primary sites (Figure 2-16) on the phenolic ring for further radical coupling forming different tri and tetramers (Ralph *et al.*, 1992). Product 20 is proposed to be a tetramer with identical linkages (*i.e.* β–5 and β–5) as evidenced by the fragmentation in which the cleavage results in identically sized β–5 ferulic acid dimer monomers. The reaction mechanism is shown in the Figure 2-16.

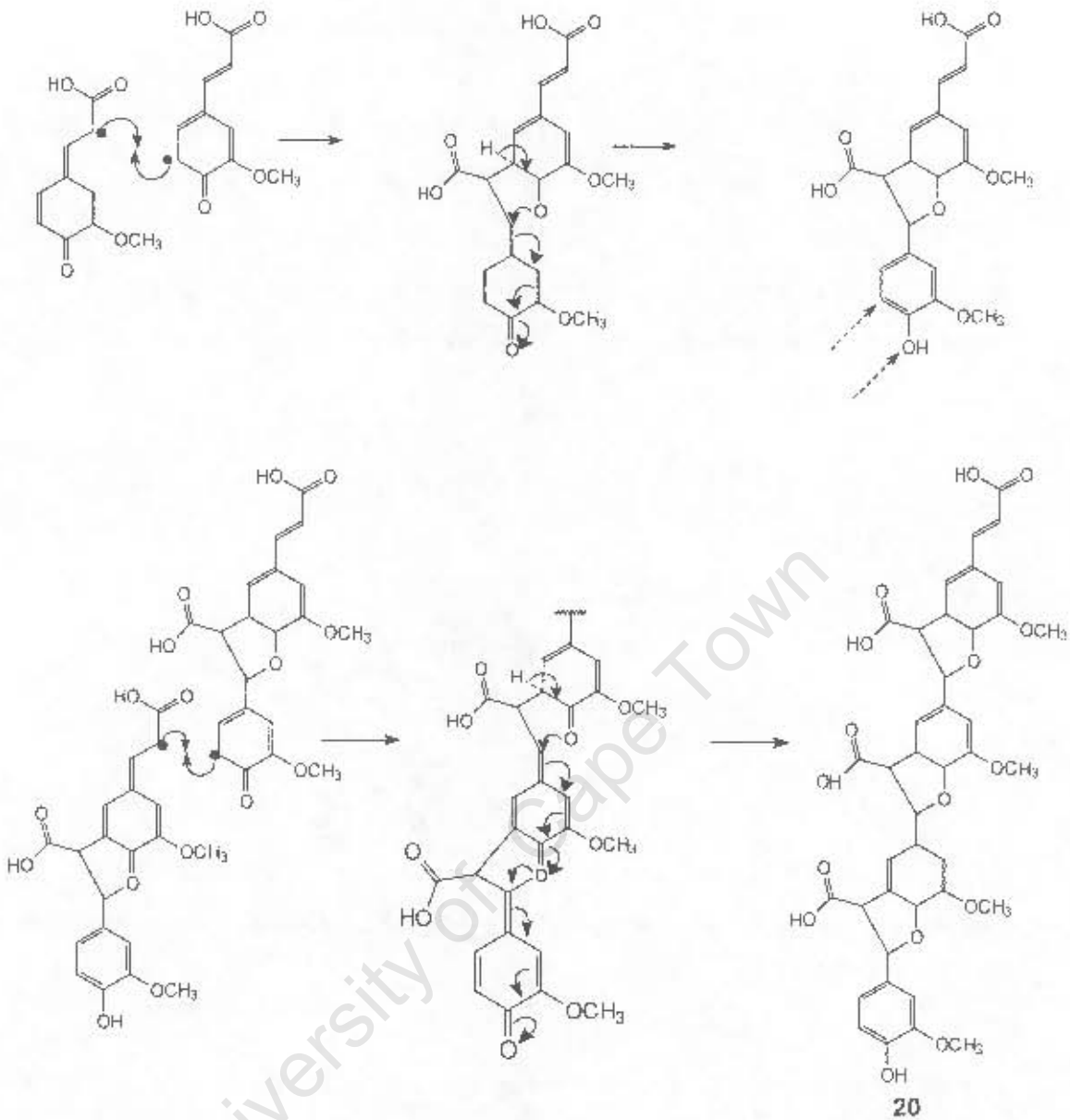


Figure 2-15. Proposed reaction mechanism for the formation of Product 20 and intermediates via 5–β linkages of ferulic acid

2.4.4.6 Analysis of product 22 and 21

Product **22** had the same molecular weight as Product **20** indicating that they were isomers. Their fragmentation patterns were similar, except for the smaller molecular weight fragments. This indicated different types of ferulic acid linkages to those proposed

for compound **20**. The dimerisation patterns of ferulic acid were outside the scope of this project, thus, the structure of Product **22** was not elucidated.

The fragmentation of product **21** is not similar to any previously reported dimer or tetramer. Its molecular weight was 745, unlike the 771 m/z obtained for products **20** and **22**. The mass spectrum of product **21** (Figure 2-17) was also very different from those of reported by Carunchio *et al.*, (2001) (P1 and P2) as well as other products isolated in this study, indicating a different type of dimer formation. Product **21** is a ferulic acid polymeric compound, but its m/z indicates the loss of a CH group (molecular weight 13) during each dimerisation (Figure 2-17). Thus, it represents another ferulic acid tetramer isomer.

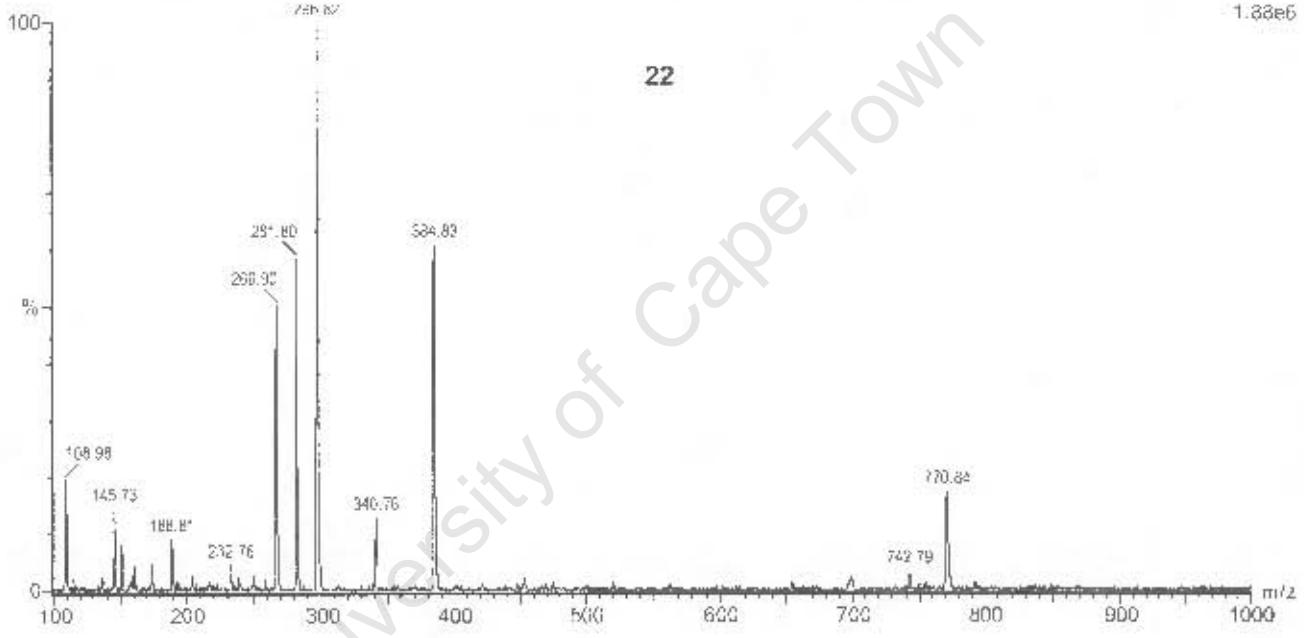


Figure 2-16. Mass spectrum of product 22, with a retention time of 17.88 min. The molecular weight was found to 771 indicating a ferulic acid tetrameric isomer.

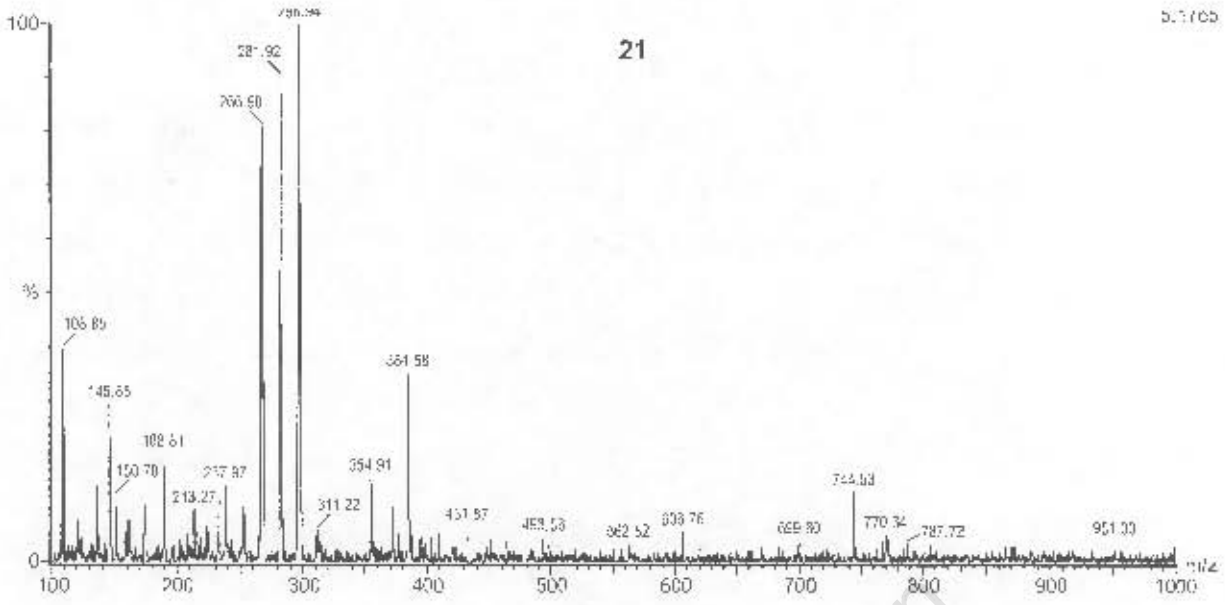


Figure 2-17. Mass spectrum of product 21. The spectrum shows a product with molecular ion of mass 745.

2.4.5 Reactions of ferulic acid catalysed by non-immobilised laccase in aqueous medium, in the presence of ABTS as mediator

ABTS is a well known laccase mediator (Thurston, 1994). As discussed in Chapter 1, mediators have been shown to increase the catalytic ability of laccases to include a wider substrate range (Riva, 2006). The work described in this section involved investigation of the effect of the presence of the laccase mediator ABTS, on the reaction of non-immobilised laccase with ferulic acid in aqueous medium.

0.5 mM ABTS was used for the reactions, and in order to shorten the reaction time, 0.6 mM (9 mmoles) ferulic acid:20 U was used. The reaction took place rapidly. Upon the addition of ferulic acid to the green solution of enzyme and ABTS in buffer, the solution lost its colour and then after 3 minutes, the solution darkened to a deep green indicative of oxidised ABTS within 5 minutes. The progression of the reaction was monitored by HPLC, which showed the formation of a single product instead of the 3 previously

observed in the aqueous reactions carried out under the same conditions without the inclusion of ABTS. Samples of this product were further characterised by LC-MS (Figures 2-18, 2-19 and Table 2-5).

The single product peak was found to be a polymeric compound of approximate m/z 588.4. From the fragmentation pattern (Figure 2-19) it was found to be a compound with a base peak of 113 and at least 7 repeats of 68 mass units. The m/z =68 repeat units could be clearly distinguished. The complexity of the mass spectra of the product obtained, precluded further conclusions on the structure of the product.

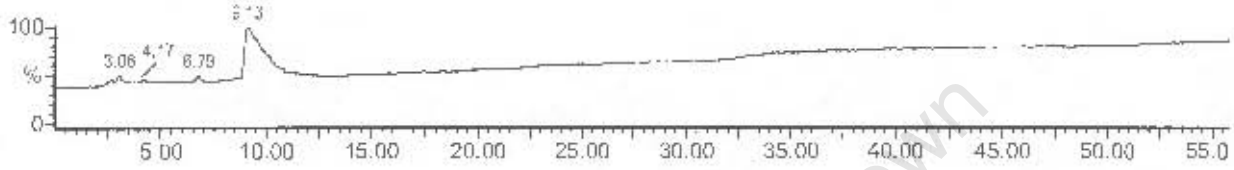


Figure 2-18. Total ion chromatogram of 0.6 mM ferulic acid reaction catalysed by 20U non-immobilised *T. pubescens* laccase in the presence of 0.5 mM ABTS after 18h reaction time.

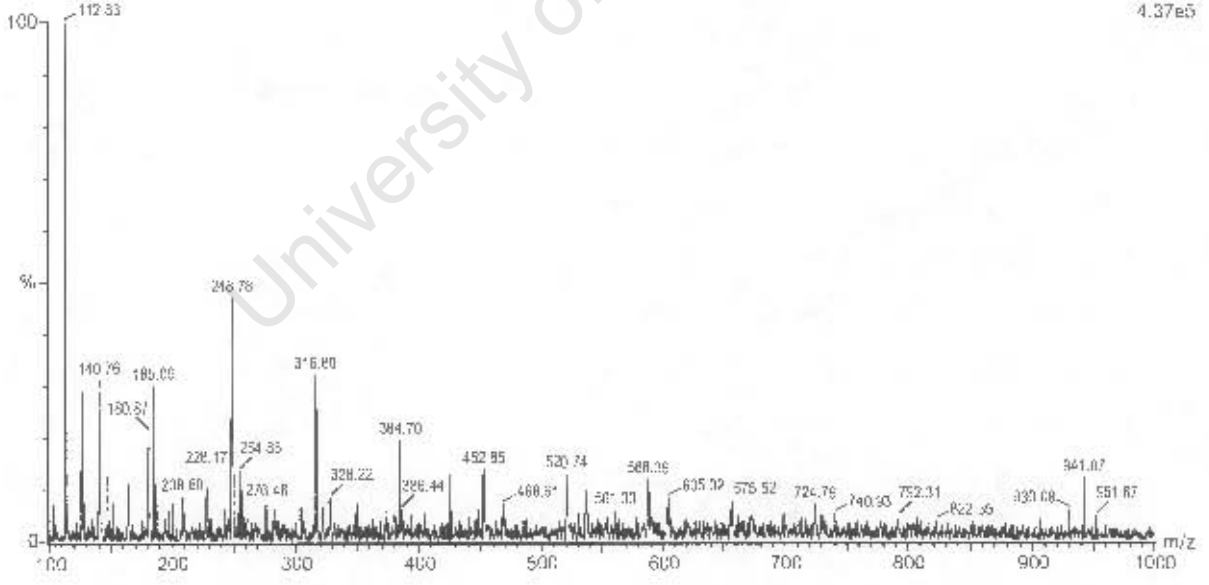


Figure 2-19. Mass spectrum of the product from 0.6 mM ferulic acid reaction catalysed by 20 U non-immobilised *T. pubescens* laccase in the presence of 0.5 mM ABTS after 18h reaction time.

Table 2-5. Masses of significant product peaks detected from the mass spectra of the product of ferulic acid reaction with non-immobilised laccase in the presence of mediator.

Mass of significant peak detected	Difference in mass
113	
181	68
249	68
317	68
385	68
453	68
521	68
588.4	67.4
942	353.6

2.4.6 Reactions of ferulic acid with immobilised laccase in aqueous medium

The effect of laccase immobilisation on ferulic acid reactions was investigated. In order to assess the effect of laccase immobilisation pH on reaction products, laccase was immobilised on Eupergit® C at different pH's. A sample of 10mg laccase immobilised in potassium phosphate buffer pH 7 on Eupergit® C biocatalyst was reacted with 1 mM (0.015 mmoles) ferulic acid and the products formed were analysed using HPLC and LC-MS. Products were formed rapidly, with the first products being detected less than 5 minutes after the addition of substrate, namely one major product (**23**) and 3 smaller intensity products. The mass spectrum of compound **23** is shown below in Figure 2-20. The product had an m/z of 385 indicating it to be a ferulic acid dimer as described by Carunchio *et al.* (2001), but otherwise had a fragmentation pattern identical to that of compound **20** produced in non-immobilised laccase reactions shown earlier. This product corresponds with product P2 detected by Carunchio *et al.* (2001).

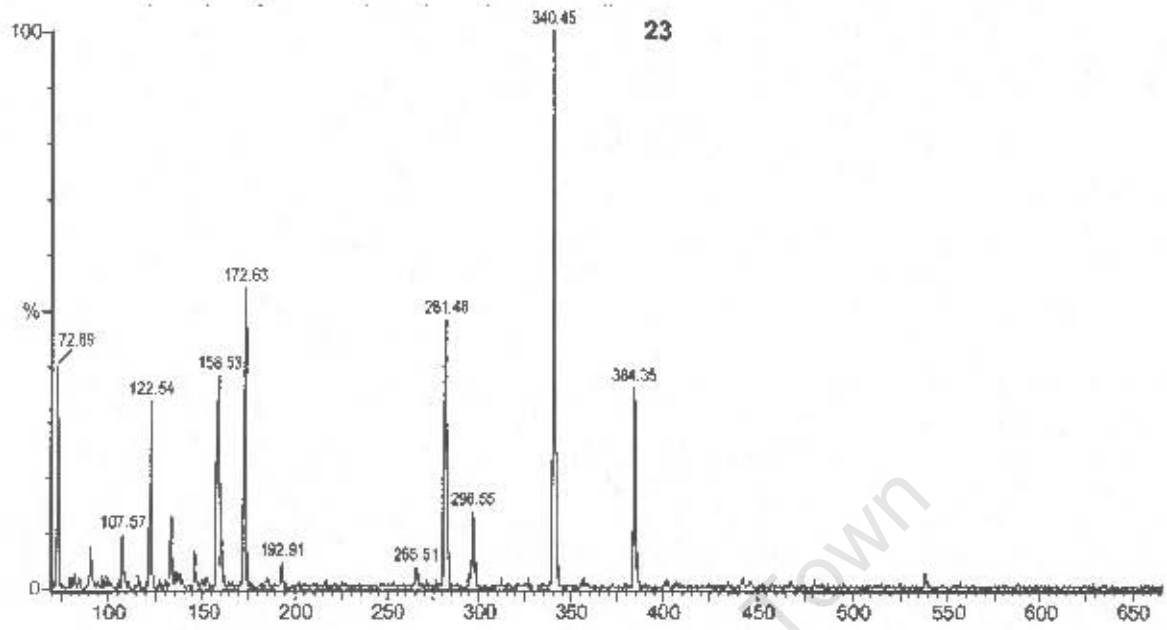


Figure 2-20. Mass spectra of initial product 23 produced by the reaction of 1 mM ferulic acid with immobilised laccase in aqueous medium.

The other product formed initially was 4-vinylguaiacol (**10**). This was detected as a low intensity product and the mass spectrum (Figure 2-21) showed the corresponding mass of 150 and fragments diagnostic of 4-vinylguaiacol (Jeong *et al.*, 2004). Therefore, Product **23** and **10** were detected as initial ferulic acid reaction products. Product **23** was also reported by Carunchio *et al.* (2001) as an initial product of the ferulic acid reaction with laccase. We report the here, for the first time the formation of 4-vinylguaiacol as a product of this reaction.

Figure 2-21. Mass spectrum of 4-vinylguaiacol (10) produced from the 1 mM reaction of ferulic acid with immobilised laccase in aqueous media.

After 5 minutes of reaction at room temperature, the total ion chromatogram of the reaction mixture showed a broad peak (Figure 2-22) and the mass spectrum of the peak labelled C, (compound **24**) on the chromatogram is shown below (Figure 2-23). The fragmentation pattern indicates a compound with a molecular weight of approximately 300 and the product was identified to be the dimer of the ferulic acid decarboxylation product, 4-vinylguaiacol, which has a molecular mass of 300. 4-vinylguaiacol is known to polymerise readily due to the reactive unsaturated vinyl moiety (Dorfner *et al.*, 2003). Based on this and literature reports, this product was identified as the 4-vinylguaiacol dimer (**24**) (Rizzi and Boekley, 1992; Newton 2005). Newton (2005) also reported the formation of two 4-vinylguaiacol dimer isomers when detected by LC-MS, and the presence of isomeric forms may explain the broad peak observed (Figure 2-22). Further mass spectral analysis of peaks A and C gave identical mass spectra, confirming this result. The proposed mechanism for the dimerisation of 4-vinylguiacol is shown below in Figure 2-24. This result is in agreement with later results obtained from GC-MS of reaction products from immobilised laccase reactions (Section 2.4.7).

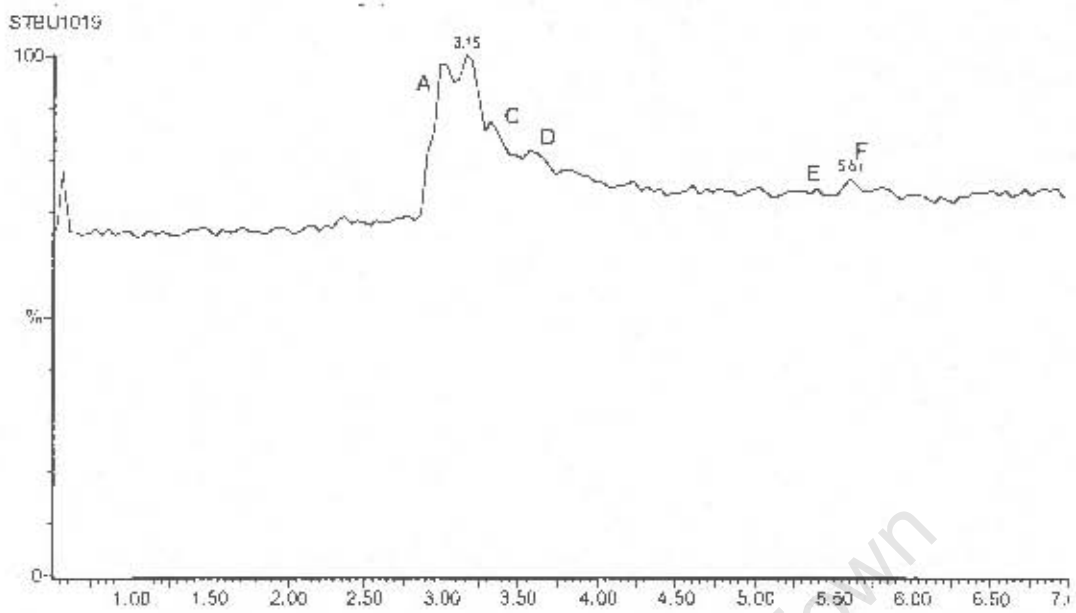


Figure 2-22. Chromatogram showing the products, after 5 min. reaction time in aqueous medium, between ferulic acid and immobilised laccase.

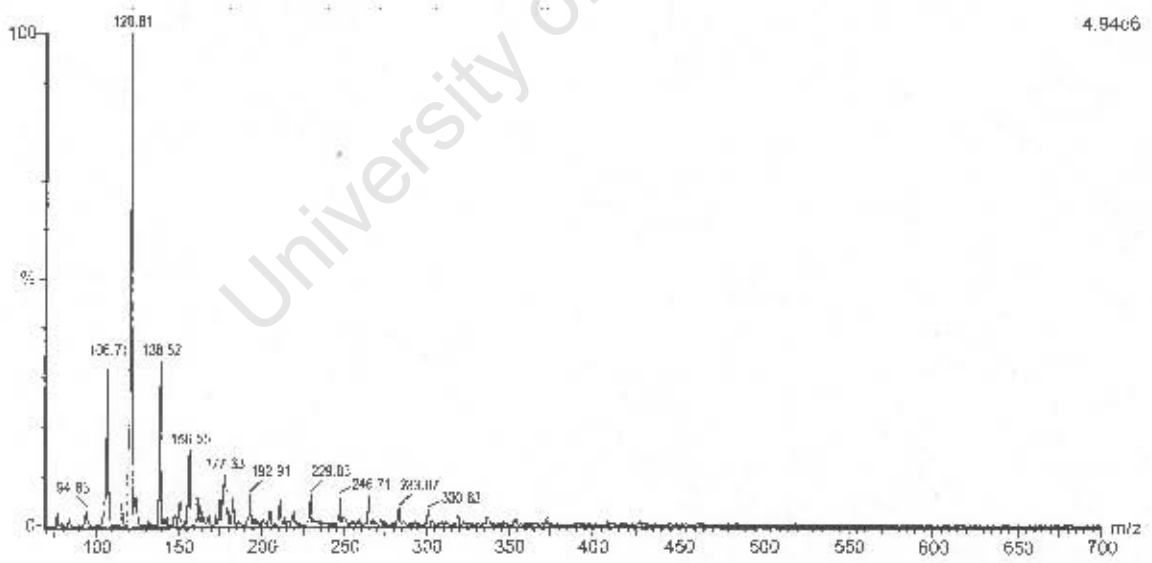


Figure 2-23. Mass spectra of one isomer of the product 24 (peak C in Figure 2-22) formed after 5 min. reaction time, of 1 mM ferulic acid with immobilised laccase in aqueous medium.

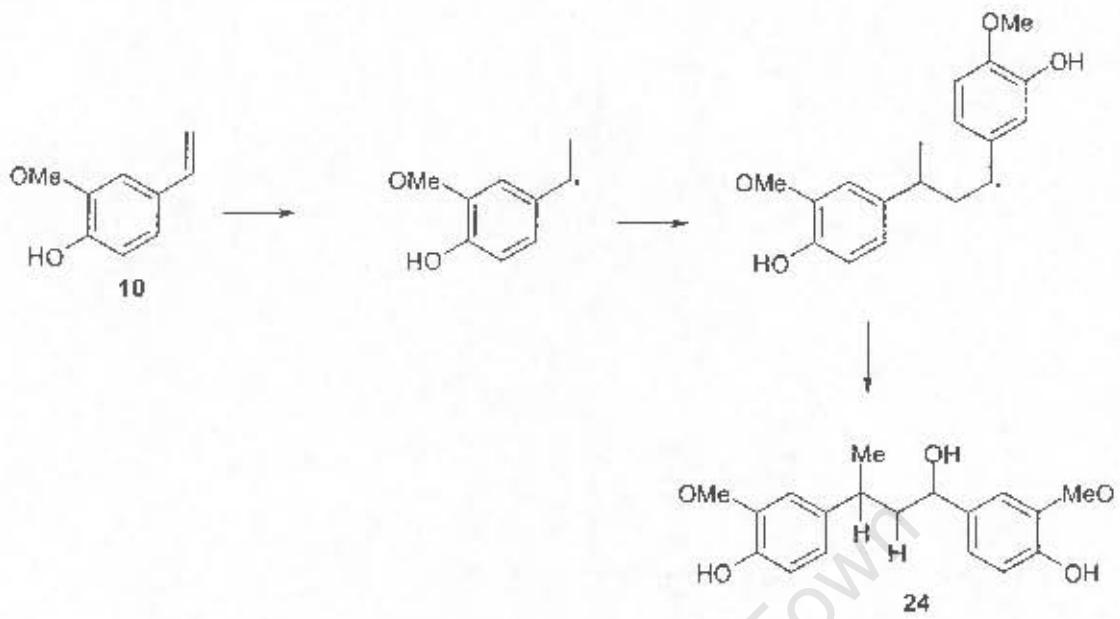


Figure 2-24. Formation of 4-vinylguaiacol dimer (24) (Rizzi and Boekley, 1992).

After 10 minutes, 2 main products were detected; one, a polymer with a mass of 347. The fragmentation pattern showed that this polymer was composed of a fragment with a molecular weight of 95 and fourteen repeats of 18 m/z (Figure 2-25). The other major product peak was identical to that found in the 5 minute sample (compound **24**).

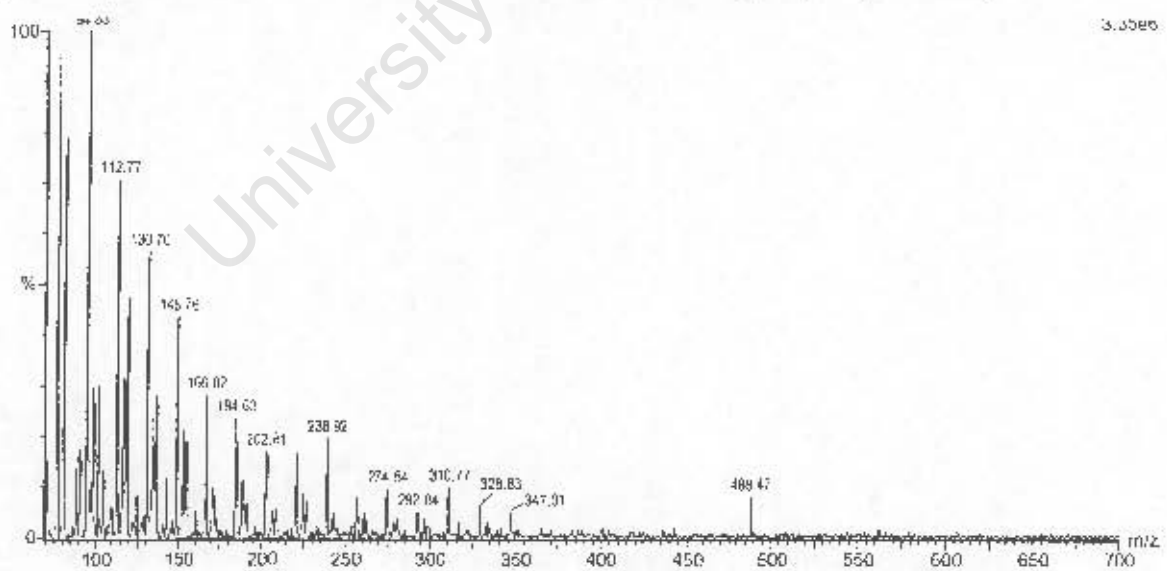


Figure 2-25. Mass spectrum of the product produced after 10 min. reaction time of 1 mM ferulic acid reaction in aqueous media catalysed by immobilised laccase.

2.4.7 Effect of solvent and immobilisation pH on reactions of ferulic acid with immobilised laccase

Since ferulic acid is not very soluble in water, the use of organic solvent media was investigated; ferulic acid reactions were conducted using immobilised laccase in 100 % reagent grade ethyl acetate, a sodium acetate buffer/ethyl acetate biphasic system, and in sodium acetate buffer.

Sodium acetate buffer (pH 5) had previously been established as an optimal buffer for laccase activity, but the effect of using enzyme immobilised at different pH was assessed in order to investigate a possible correlation between the pH of immobilisation and the type of products formed for our biocatalytic synthesis of the products. The pH studies were based on the fact that enzymes used in organic media have been found to have "pH memory". This is a phenomenon in which enzymatic catalytic activity is affected by the pH of the last aqueous solution to which they were exposed (Zaks and Klibanov, 1985). The progress of the reactions was monitored by HPLC and then analysed by GC-MS.

2.4.7.1 HPLC analysis of reaction products produced from ferulic acid with immobilised laccase in different media

In reactions conducted using ethyl acetate and /or sodium acetate buffer, 10 mM (0.1 mmoles) ferulic acid was reacted with 100 mg (approx. 300 U) immobilised laccase, and samples were taken periodically for analysis by HPLC. It was observed from HPLC analysis that most of the ferulic acid was not utilised even after 60 minutes reaction time in all media. Tables 2-6 and 2-7, below show a comparison of the peak areas identified for different products with specific retention time after an approximately 18 and 36 hours reaction time respectively.

Table 2-6. Products formed after 18h reaction of ferulic acid with immobilised laccase in various solvent media. The different products were identified by their HPLC retention times (Rt).

			Peak intensities(uV*sec) using laccase immobilised at different pH's		
Reaction	**Phase**	**Peak identity**	**pH 7**	**pH 5.7**	**pH 2**
Ethyl acetate only	Organic solvent	Ferulic acid	50429478	93212915	68436236
		Rt =11.56	6546597	-	-
Biphasic reaction; ethyl acetate and sodium acetate buffer (pH 5)	Organic solvent	Ferulic acid	108000000	119300000	81624678
		Rt =30.63	3548776	3450587	3582200
	Aqueous phase	Ferulic acid	14530308	1615718	9992944
		Rt = 22.99	6866507	-	-
		Rt = 26.62	-	6300457	7548934
		Rt = 30.76	3568469	3718109	3732940
		Rt = 42.99	259211	-	-
		Rt = 45.13	-	303474	297482
Aqueous reaction	Aqueous	Ferulic acid	72502784	94130636	91469843
		Rt = 23.52	6731724	-	-
		Rt =28.11	-	6478389	-
		Rt =32.6	-	-	6998261

Table 2-7. Products formed after 36h reaction of ferulic acid with immobilised laccase in various solvent media. The different products were identified by their HPLC retention times (Rt).

			Peak intensities (uV*sec) using laccase immobilised at different pH's		
Reaction	**Phase**	**Peak identity**	**pH 7**	**pH 5.7**	**pH 2**
Ethyl acetate only	Organic solvent	Ferulic acid	-	-	51328489
		Rt = 13.14	10028130	74857158	-
Biphasic reaction; ethyl acetate and sodium acetate buffer (pH 5)	Organic solvent	Ferulic acid	-	-	50360051
		Rt = 13.73	62120153	81098732	-
		Rt =22.99	4693799	5269446	-
		Rt = 25.99	-	-	4947096
		Rt = 30.63	414381	-	-
		Rt = 59.05	1685112	-	-
	Aqueous phase	Ferulic acid	8772534	10555774	5456364
		Rt = 4.61	-	3642102	-
		Rt =8.57	-	-	4453633
		Rt = 22.99	5575742	-	-
		Rt = 25.44	-	5185177	458721
		Rt = 30.76	423133	-	-
		Rt = 36.35	-	-	441392
Aqueous reaction	Aqueous	Ferulic acid	-	79055001	75714073
		Rt = 6.68	-	3276601	-
		Rt =10.02	-	-	3441939
		Rt =13.93	57178323	-	6998261

One predominant reaction product was noted for the reactions catalysed by immobilised laccase, which was less polar than ferulic acid (Rt – 13.14 for 36h reactions). This was the only product formed when the reaction was performed in neat ethyl acetate when catalysed by laccase immobilised at pH's of 5.7 and 7 (Figure 2-26). This same product was formed as well in reactions performed in the biphasic media and only in the aqueous reaction catalysed by laccase at pH 7. This product was confirmed by GC-MS analysis in the following section as 4-vinylguaiacol (**10**).

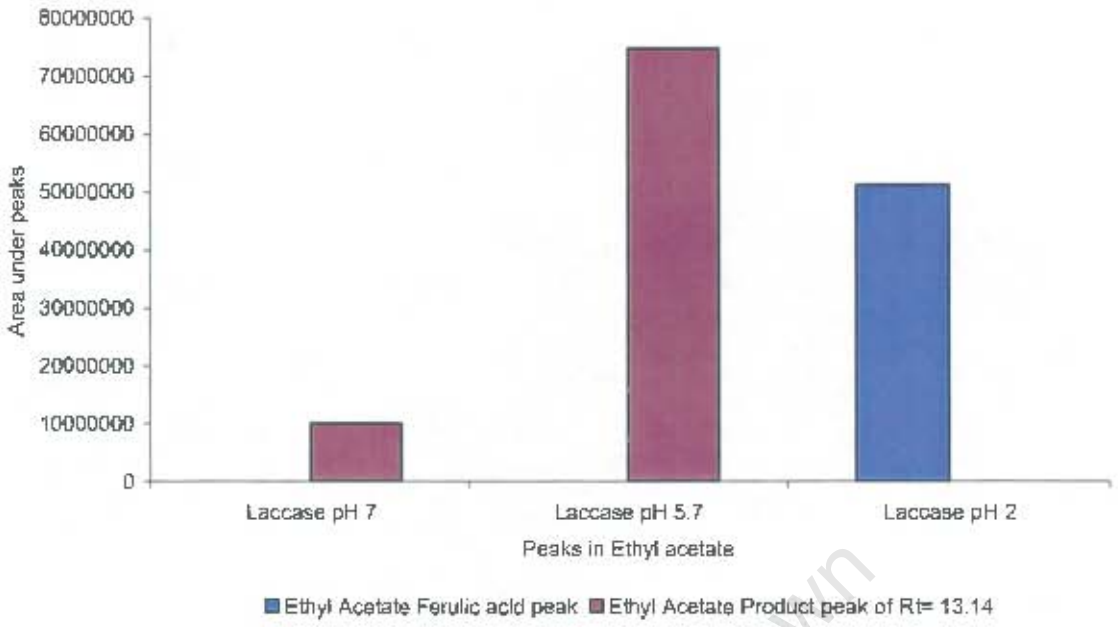


Figure 2-26. Products formed after 36h reaction of ferulic acid with immobilised laccase in ethyl acetate

When the reaction was catalysed by laccase immobilised at pH 7 in sodium acetate buffer, the same product (Rt=13.14 min.) discussed above was observed as well as small amounts of another product with a retention time of 49 minutes. Previous reactions of ferulic with non immobilised laccase yielded isomers of ferulic acid tetramers in section 2.4.4.4 and the less polar compound is presumed to be a ferulic acid tetramer as observed when non-immobilised ferulic acid was used. When the reaction was catalysed by laccase immobilised at pH 5.7 and 2, very little conversion of ferulic acid was noticed after 36h. Most of the ferulic acid was left unconverted, products with higher polarity than ferulic acid compounds being detected (Rt = 6.68 and 13.93 min. respectively, Figure 2-27). These possibly were 4-vinylguaiacol dimers (isomers of product **24**) as detected previously in Section 2.4.6.

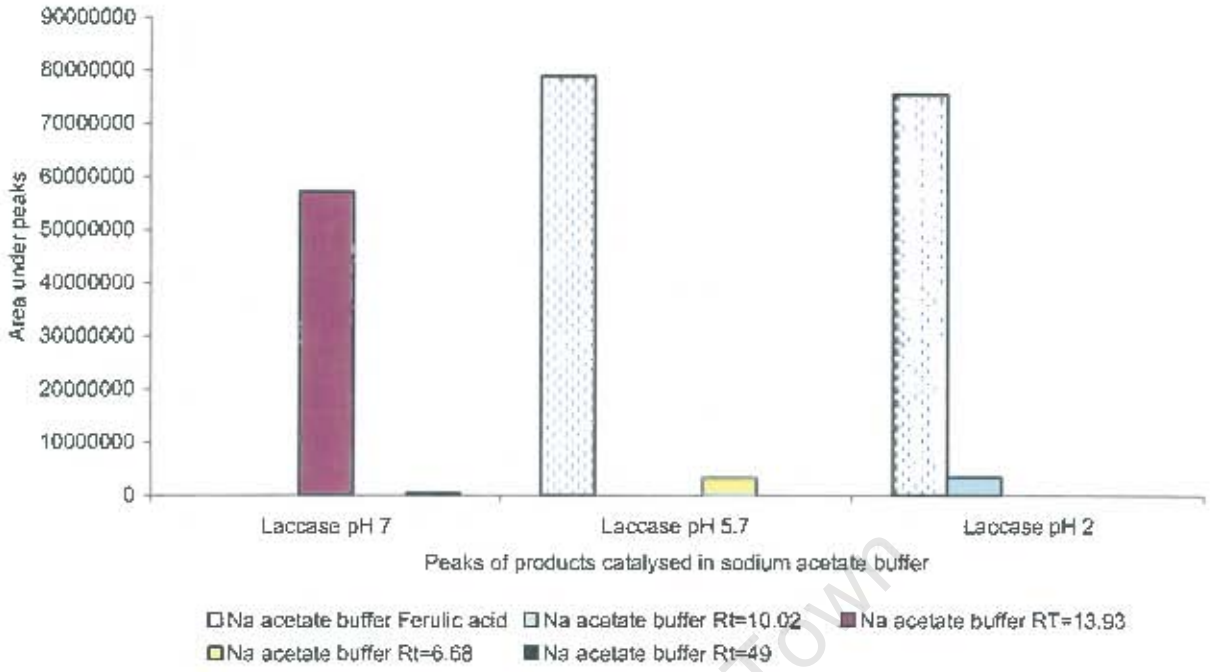


Figure 2-27. Products formed after 36h reaction of ferulic acid with immobilised laccase in sodium acetate

The product with the retention time of 13.73 minutes formed in ethyl acetate previously, was detected as the predominant product of the reactions catalysed by laccase immobilised at pH 7 and 5.7 in biphasic medium. Other products were detected as well with higher retention times than ferulic acid (Figure 2-28). These were also presumed to be ferulic acid polymers. Several of the polymeric products were formed, and their formation was found to reflect the pH's in which the laccase was immobilised in. It was interesting to note that not all the ferulic acid was utilised in the biphasic reactions. Ferulic acid catalysed by laccase immobilised at pH 5 was barely utilised, and the same applied for reactions catalysed by laccase immobilised at this pH in sodium acetate buffer.

Ferulic acid is more soluble in the aqueous phase as it is a polar molecule. Lee *et al.*, (1998) calculated the ferulic acid partition coefficient in ethyl acetate and phosphate buffer to be 0.8 and for 4-vinylguaiacol to be 120. This indicates that in an aqueous: ethyl

acetate two phase system, most ferulic acid would be readily transferred to the aqueous phase and 4-vinylguaiacol in the organic phase (Lee *et al.*, 1998). The findings reported here concur with the above. The product that was identified as 4-vinylguaiacol was found in the organic phase of the reactions that were performed in biphasic media and its synthesis from ferulic acid was enhanced in ethyl acetate. The polymers were also found to be predominantly localised in the aqueous phase of the reaction. The formation of ferulic acid polymers was enhanced in aqueous conditions. This partitioning of the products was observed when the products were tested for antioxidant activity described in section 4.3.5.6.

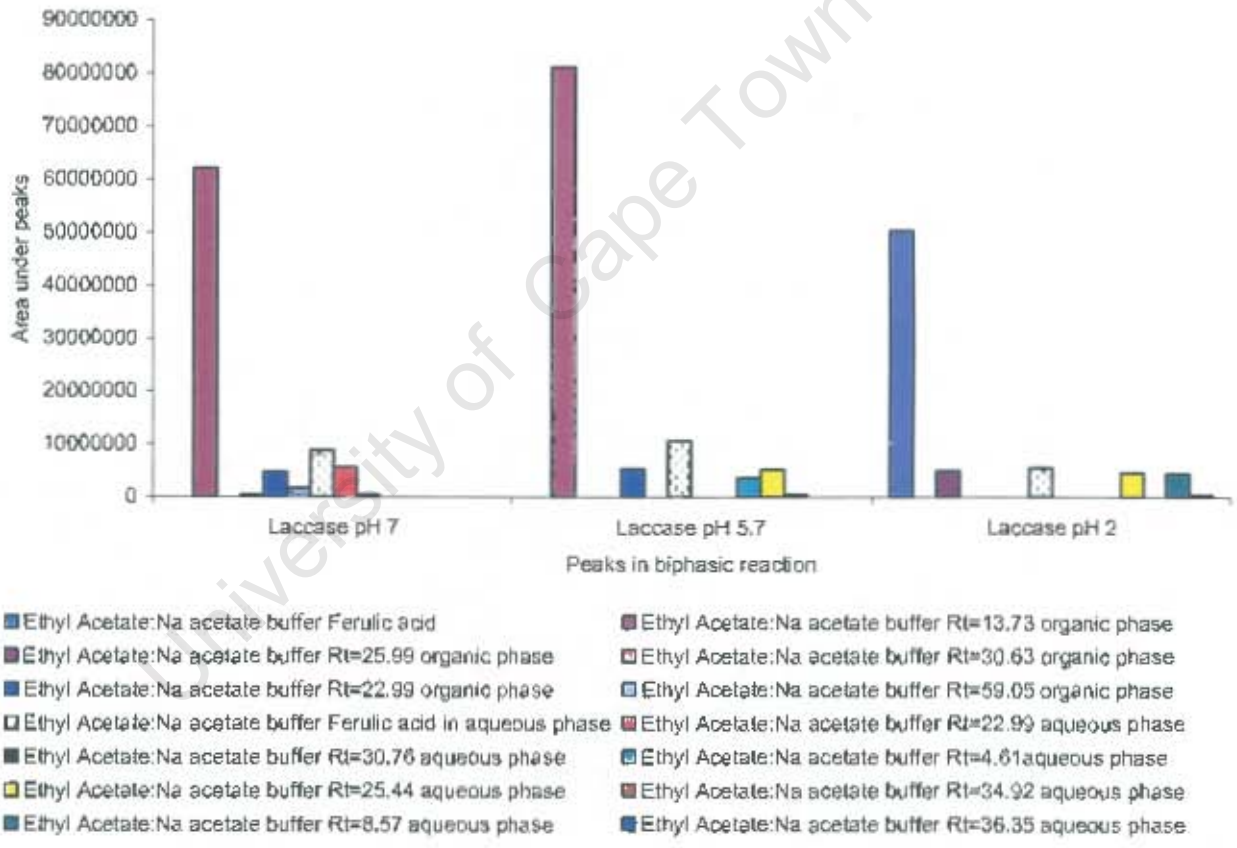


Figure 2-28.Products formed after 36h reaction of ferulic acid with immobilised laccase in biphasic sodium acetate-ethyl acetate medium

These results demonstrate that formation of specific products was affected by the reaction medium. These findings agree with the observation by Intra *et al.* (2005) that laccases are amenable to 'medium engineering,' a phenomenon only previously noted for hydrolase type enzymes (Riva, 2006). A more diverse set of products was formed in the biphasic system, followed by the aqueous buffer system and more specific products were formed in the organic solvent system. It was also observed that the pH at which the laccase was immobilised had an effect on the reaction products formed.

2.4.7.2 GC-MS analysis of reaction products produced from ferulic acid with immobilised laccase in different media

The TLC results of the ferulic acid non-immobilised laccase reaction described in section 2.4.4.1 showed the formation of 2 types of products: a product that was more polar than ferulic acid and secondly, less polar polymeric products (Figure 2-7). These polymers were identified as ferulic acid tetramers (**20, 21,** and **22**) by LC-MS in section 2.4.4.4. In section 2.4.6 preliminary reactions of ferulic acid with laccase immobilised at pH 7 yielded 4-vinylguaiacol (**10**) and a ferulic acid dimer, product **23.** From the HPLC analysis of the reactions of immobilised laccase and ferulic acid, described in the previous section, one major, more polar than ferulic acid product (Rt. of approx. 13 minutes), was detected and lower concentration. Less polar than ferulic acid products were observed as well after 18 and 36h reaction time (Tables 2-6 and 2-7). The identity of the less polar predominant product was unidentifiable using HPLC analysis based on retention times of standard compounds. Therefore, GC-MS with a NIST library database was used to identify the compound. The product was identified to be 4-vinyl-2-methoxy phenol, commonly named 4-vinylguaiacol. Figure 2-29 shows the gas chromatogram of the product of the reaction catalysed by pH 5.7 immobilised laccase in ethyl acetate. The identity was confirmed (93 %) using the NIST database (Figure 2-30). The negative control of the reaction (no enzyme added) showed no reaction products.

Table 2-8. Summary of the products formed from the ferulic acid reactions with laccase under different reaction conditions.

Ferulic acid with laccase reaction conditions	Products formed	Comments on products formed	General comments
Aqueous medium with non-immobilised laccase	Formation of product 20, ferulic acid tetramer and 2 unknown tetrameric isomers		
Aqueous medium with non-immobilised laccase with mediator	1 predominant product 113 plus 7 repeats of 68	Structure not resolved	
Aqueous medium with immobilised laccase	Initial product formed was ferulic acid dimer **23**, and **10** formed; after 5 min. isomers of **24** after 10 min. compound with m/z of 347 and **24**	Immobilisation resulted in formation of mainly one product, a non- polymer and lower concentrations of polymeric compounds	4-Vinylguaiacol formed in only laccase immobilised at pH 7,ferulic acid polymers in others; Putative 4-vinylguaiacol dimers formed
Biphasic medium with immobilised laccase	4-vinylguaiacol, 4-vinylguaiacol dimers + ferulic acid polymers		4-Vinylguaiacol produced in reactions catalysed by laccase immobilised at pH 7 and 5.7 only. Ferulic acid polymers detected.
Organic medium with immobilised laccase	4-vinylguaiacol only		4-Vinylguaiacol produced in reactions catalysed by laccase immobilised at pH 7 and 5.7 only

2.4.8 Discussion on the formation of 4-vinylguaiacol from ferulic acid catalysed by laccase

The mechanism for the decarboxylation of ferulic acid to form 4-vinylguaiacol is not well understood (Rizzi and Boekley, 1992; Peleg *et al.*, 1992; Rosazza *et al.*, 1995; Newton, 2005). It was not anticipated that the formation of 4-vinylguaiacol from ferulic acid would be catalysed by ferulic acid. However, this was observed and the following is postulated as a possible explanation.

The decarboxylation of β,γ-unsaturated carboxylic acids are known to take place easily. This can be achieved thermally (Fiddler *et al.*, 1967), by acid hydrolysis (Johnson and Heinz, 1949) or by biotransformation (Karmakar *et al.*, 2000; Takemoto and Achiwa 1999). Ferulic acid is an α,β-unsaturated acid which can isomerise to a β–γ system via shift of a proton, and is therefore readily decarboxylated, forming the styrene (Figure 2-33, March, 1985; Rizzi and Boekly, 1992). Fiddler *et al.* (1967) suggested a free radical chain mechanism for ferulic acid decarboxylation, which has been disputed by Rizzi and Boekly, (1992), but concurred by Rosazza *et al.* (1995), and Rodriguez-Evora and Schepp (2005) in the biosynthesis of lignans. It is noteworthy that laccase is involved in lignin biosynthesis and catalyses its reactions via free radicals. It is postulated that laccases can catalyse the decarboxylation of ferulic acid to form 4-vinylguaiacol via a free radical mechanism, which is demonstrated by our results.

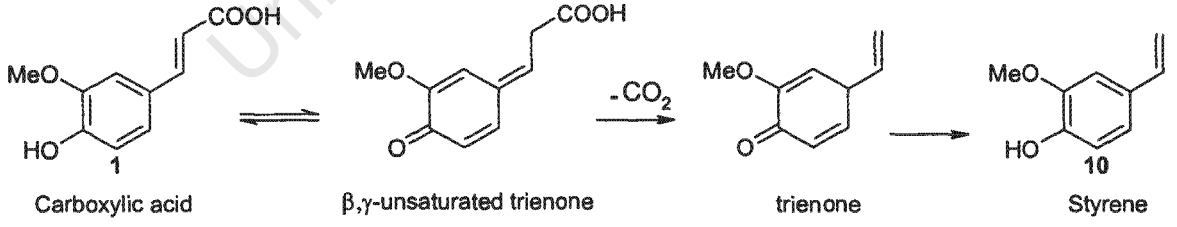


Figure 2-32. The decarboxylation of ferulic acid to 4-vinylguaiacol showing isomerisation of the carboxylic acid to form an unsaturated β,γ-trienone which lose carbon dioxide easily (Rizzi and Boekly, 1992).

2.4.9 Conclusions

The synthesis of vanillin and vanillic acid from ferulic acid and laccase under the conditions investigated in this study was not successful. Instead, 4-vinylguaiacol and ferulic acid polymers were formed. The type of polymers formed varied depending on the reaction medium, as polymerisation is influenced by solvents and matrix effects (Ralph *et al.,* 1992) and the polymers formed with immobilised laccase were found to be different to those found using non-immobilised enzyme. Specifically, ferulic acid tetramers were formed when the reaction was catalysed by non-immobilised laccase and dimers were formed when the enzyme was immobilised. 4-vinylguaiacol was solely formed when the reaction was catalysed in organic solvent. Hence immobilisation of laccase on Eupergit C resulted in smaller molecular weight products being formed from ferulic acid. This may be due to the nature of the covalent bonding that takes place between the enzyme and solid support, which may alter the enzyme active site.

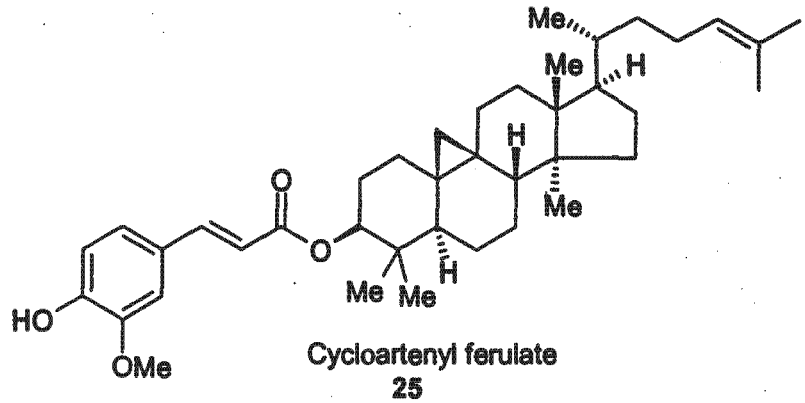
A correlation between medium and products formed was demonstrated, as the formation of certain products was favoured in certain media. Generally, ferulic acid reactions with laccase reactions in aqueous medium were shown to result in ferulic acid tetramers. Different tetrameric regioisomers were detected and their antioxidant activity is described in a later chapter (Chapter 5). The reactions in organic solvents resulted in the formation of higher quantities of 4-vinylguaiacol. The ferulic acid polymers have potential use as antioxidants and their antioxidant activity is assessed in Chapter 4.

3 The use of lipases to synthesize bio-active, ferulate esters

3.1 Introduction

This chapter describes the biocatalytic synthesis of ferulic acid steroid derivatives similar in structure to natural triterpene derivatives of ferulic acid, such as cycloartenyl ferulate (25). The objective in the present study was to confer increased biological activity (for example antioxidant activity) to ferulic acid by synthesising steroid derivatives of ferulic acid which would have structures related to those of natural biologically active compounds.

Alkyl esters of ferulic acid, such as octyl ferulate, have been observed to have higher antioxidant activity than the acid itself (Kikuzaki *et al.*, 2002). Their inhibition of the oxidation of linoleic acid was reported to be equivalent to that of butylated hydroxytoluene (BHT), a well-known antioxidant food additive. Further, the triterpene alcohol monoesters of ferulic acid, such as 24-methylenecycloartenyl ferulate and cycloartenyl ferulate (25) (Figure 3-1), also display antioxidant activity (Yagi and Ohishi, 1979) and were shown to inhibit oxidation more effectively than ferulic acid (Kikuzaki *et al.*, 2002). Similarly, γ-oryzanol, a rice bran extract consisting primarily of cycloartenyl ferulate and 24-methylenecycloartenyl ferulate, is widely used in the cosmetic industry as an antioxidant (Graf, 1992; Cicero and Gaddi, 2001). Sterol ferulates have also been observed to have various other pharmaceutical applications, such as anti-inflammatory effects (Akihisa *et al.*, 2000), modulation of pituitary secretion, anticancer activity (Berger *et al.*, 2004) for antiulcerogenic effects and treatment for hyperlipoproteinanemia (Cicero and Gaddi, 2001). Thus, it was worth pursuing a biocatalytic route for the synthesis of ferulate derivatives, similar to cycloartenyl ferulate with potential biological activity, in this study.



Cycloartenyl ferulate
25

Figure 3-1. Structure of cycloartenyl ferulate, the major component of rice bran oil, γ-oryzanol.

Biocatalysis using lipases has enabled the selective modification of various steroid functional groups (Riva and Klibanov, 1988; Riva *et al.*, 1989; Bertinotti *et al*, 1994).

3.2 The use of lipases in organic synthesis

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are the most commonly used biocatalysts in organic synthesis (Itoh *et al.*, 1997; Gotor, 1999; Theil, 1995; Jaeger and Eggert, 2002). This is due to several of their properties, listed below:

- Lipases are active in organic solvents. Many organic synthesis reactions involve the use of hydrophobic, non-polar substrates that are not miscible with or soluble in water. The ability of lipases to maintain catalytic activity in organic solvents (Zaks and Klibanov, 1984) makes them very useful in such reactions.
- Lipases usually display chemo-, stereo- and regioselectivity. These characteristics are often critical in the synthesis of fine chemicals, for example in drug design and in the flavour industry, due to the complex and chiral nature of these products. In general, only one of the two enantiomers of a drug is pharmaceutically functional (the other may be toxic) or, alternatively, often only one isomer of a compound is active as a flavourant (for example, only one out of eight possible isomers of menthol has the desired menthol effect) (Clark, 1998).

- Lipases are readily available in large quantities. Many lipases are readily available from bacteria and fungi, and may be produced as extracellular enzymes which aids their extraction. Using molecular and bioprocess engineering techniques such as heterologous protein expression and fermentations, lipases can be produced in large scale (Jaeger and Eggert, 2002).
- Lipases are some of the most well studied enzymes. The molecular sequences of many lipases have been elucidated, along with their crystal structures. From this knowledge, many rational protein-engineering strategies have been designed (Jaeger and Eggert, 2002).
- Lipases do not require cofactors and neither do they catalyse side reactions, in general (Jaeger and Eggert, 2002).

Lipases are defined as "carboxyesterases which naturally catalyse the hydrolysis and synthesis of long chain acylglcerols with triacylglcerol being the standard substrate" (Jaeger and Eggert, 2002). These enzymes catalyse the hydrolysis of carboxylic acid esters or the synthesis of diverse esters and amides when applied in organic solvents (Figure 3-2), the latter being their most common biocatalytic application. A number of natural biologically active compounds are esters, and lipases are extensively utilised in their synthesis (Davies and Boyer, 2001).

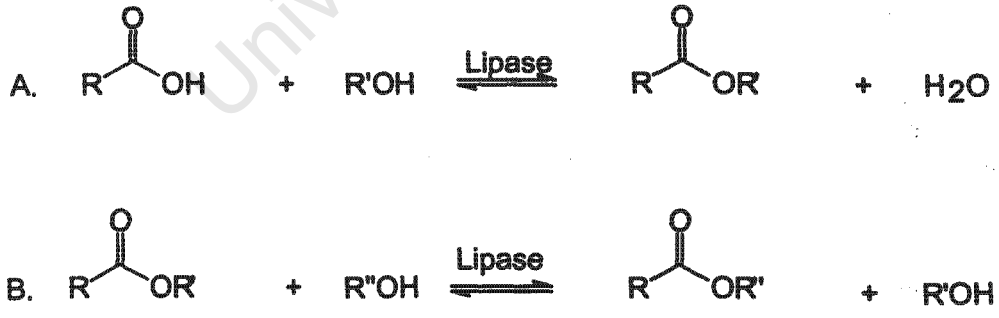


Figure 3-2. Reversible lipase esterification (A) and transesterification (B) (adapted from Reetz, 2002).

Lipases have been used widely for the regioselective acylation of nucleosides (Moris and Gotor, 1993), flavonoids (Riva *et al.*, 1990; Enaud *et al.*, 2004) sugars and steroids

(Bertinotti *et al.*, 1994 and Riva *et al.*, 1990) amongst other compounds. In the case of the latter two groups, the acylations were selective for the primary hydroxyl groups.

3.2.1 Properties of lipases

Lipase esterifications in organic solvent are reversible (Reetz, 2002), and a large excess of the acyl donor is necessary in order to increase the conversion and reaction rate of acylations. The most common acyl donors possess good leaving groups (activated acyl donors) in order to make the acyl-transfer reaction irreversible. These donors include vinyl esters, vinyl carbonates and trifluoroethyl esters (Usyatinsky *et al.*, 2003, illustrated in Figure 4-3).

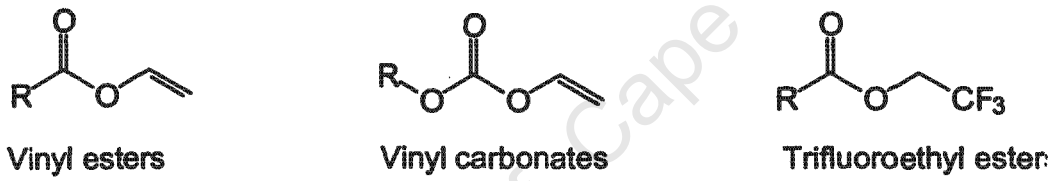


Figure 3-3. Activated acyl donors (From Usyatinsky *et al.*, 2003).

Lipases do not follow typical Michaelis-Menton kinetics which depends on the substrate concentration. Lipase activity increases sharply as the substrate concentration is increased beyond its solubility limit and they do not hydrolyse the substrate when its concentration is less than the "critical micellar concentration" (CMC). This phenomenon is known as 'interfacial activation' (Faber, 1995). Uniquely, they catalyse hydrolysis reactions as the enzyme conformation changes and a "lid" shielding the active site is moved when it comes in contact with water insoluble substrates at the water-lipid interface. Lipase active sites are typically composed of a triad of serine, histidine and aspartate (Reetz, 2002; Faber, 1995).

The ability of lipases to catalyse transesterifications and the pharmaceutical applications of sterol ferulates purified from rice bran oil, as described here, led to the hypothesis, in the present study that the biocatalytic conversion of ferulic acid to sterol ferulates would

be possible using lipases. This has not been reported previously, but the ability of lipases to perform transesterification reactions is well established. This chapter describes the novel biocatalytic synthesis of biologically active compounds with structures similar to those found in γ-oryzanol, from ferulic acid. In order to conduct transesterifications using ferulic acid by lipase, the activated acyl donor of ferulic acid, vinyl ferulate, was synthesised. An acyl donor, trifluoroethyl cinnamate synthesised previously was also used for acylations as it was shown to be a good lipase acyl donor previously (Ottolino *et al.*, 1990). The compound was readily available and as a result, was used in conjunction with the chemically synthesised vinyl ferulate. The biocatalysts used in the present study were the commercial immobilised lipases from *Candida antarctica* and *Candida rugosa*.

3.3 Materials and Methods

The work described in this chapter was conducted in collaboration with Dr Sergio Riva, Istituto di Chimica del Riconoscimento Molecolare, CNR, Milano, Italy.

3.3.1 Materials

Enzymes were purchased from Sigma-Aldrich, South Africa. All chemicals were purchased from Sigma-Aldrich, including the silica gel 60 (TLC plates and powder) used for purification. Solvents were purchased from Merck, South Africa or Italy and were HPLC grade.

3.3.2 Acylation of ferulic acid to produce vinyl ferulate

Ferulic acid vinyl ester was prepared via the vinyl exchange reaction of vinyl acetate and ferulic acid as previously described by Gao *et al.* (2001). Ferulic acid (10 mmol) was added to a three-necked flask with 15 mL vinyl acetate (0.16 mol), 66 mg (4 % v/v) mercury acetate and 10 ml Tetrahydrofuran, THF. The reaction mixture was stirred under nitrogen for 30 minutes and 0.04 mmol (2 µL) sulphuric acid was added to start the reaction. The reaction mixture was heated to 40°C with stirring for 12h, and the reaction was terminated by the addition of excess sodium acetate (20 mg). The progress of the reaction was monitored regularly using thin layer chromatography (TLC), eluted with petroleum ether /ethyl acetate (4:1 v/v). The solvent was evaporated and the solid residue was subjected to silica gel chromatography using the same solvent composition.

3.3.3 Biocatalytic reactions

3.3.3.1 Enzymatic transesterification of vinyl ferulate or trifluoroethyl cinnamate with n-octanol

Vinyl ferulate or trifluoroethyl cinnamate (50 mg/μL) were reacted with 10 μL octanol in 1 mL tert-butyl- methyl ether. *Candida antarctica* B lipase (5 mg) was added and the reaction was stirred at 45°C. Samples were taken at regular intervals to monitor the reaction. The solution was filtered to remove the enzyme and products were identified using GC-MS.

3.3.3.2 GC-MS analysis

A Finnigan-Thermo GC-MS instrument was used, with helium as the carrier gas. Electron impact MS was performed and the GC column used was an HP1 MS column (30 m x 250 μm x 0.25 μm). The parameters were:

Initial temperature	50°C
Final column temperature	250°C
Ramp rate	5°C/min
Injector temperature	250°C

3.3.3.3 Enzymatic transesterification of vinyl ferulate or trifluoroethyl cinnamate with arbutin

Arbutin (74 mM) and vinyl ferulate (213 mM) or trifluoroethyl cinnamate, were reacted in acetonitrile (3 mL) at 45°C with shaking. 60 mg immobilised *C. antarctica* B lipase (Novozyme 435) was used as the biocatalyst, and the progress of the reaction was monitered by TLC. At the end of the reaction, the enzyme was filtered off and the solvent evaporated. The resulting solid residue was purified using silica gel chromatography. The mobile phase was ethyl acetate:methanol: water (10:0.5:0.1, v/v). Substrates and products were visualised at 254 nm and/or by plates treated with phosphomolybdate reagent

[$(NH_4)_6Mo_7O_{24}.4\ H_2O$, 42 g; $Ce(SO_4)_2$, 2 g; H_2SO_4 conc., 62 mL; made up to 1 L with deionized water).

3.3.3.4 Acetylation of steroid by vinyl acetate

A modification of the method described by Bertinotti *et al.* (1994) was used. 5 mg (12 mM) dihydrocholesterol was dissolved in 900 µL tert-butyl-methyl ether. Vinyl acetate (100 µL) and *C. antarctica* B lipase (5 mg) were added and the reactions were performed at 45°C with shaking. The progress of the reaction was monitored by TLC using chloroform/methanol (10:0.1, v/v) as mobile phase, using an authentic sample of 3-*O* acetyldihydrocholesterol as a reference.

3.3.3.5 Synthesis of sterol ferulates

Vinyl ferulate (227 mM) was reacted with steroid (26 mM dihydrocholesterol, 5α-androstane-3β, 17β-diol, or β-estradiol) in tert-butyl-methyl ether. An excess of vinyl ferulate was used to drive the synthesis. The biocatalysis was attempted with four different lipases, namely *C. antarctica* B (10 mg/mL), *C. rugosa* (100 mg/mL), *Chromobacterium viscosum* (100 mg/mL) and *Pseudomonas* sp. lipase (75 mg/mL), used at 45^{0}C with shaking. After filtration and solvent evaporation, the residue in each case was purified by silica gel chromatography using petroleum ether: ethyl acetate, (10:1.5, v/v) as the mobile phase. The reaction was monitored by TLC at regular intervals and Komarowsky reagent was used for the visualisation of the steroids (Grant, 1963).

3.3.4 NMR analysis

^{1}H-NMR (300 and 400 Hz) analysis was recorded on a Bruker AC-300 (Milano, Italy) or Varian Unity Spectrometer (Department of Chemistry, University of Cape Town). Tetramethylsilane (TMS) was used as an internal standard, and the chemical shifts (δ) are given in ppm, relative to TMS.

Vinyl ferulate (26). 1H-NMR (Chloroform-d) δ:7.74 (1H, d, J=16 Hz, *H*C=CH-C=O), 7.44(1H, dd, J=6 Hz, 14, O-C*H*=CH_2), 7.12(1H, dd, J=2 Hz, 8 Hz, Ar-H), 7.05 (1H,d, J=2 Hz, Ar-H), 6.94(1H,d, J=8 Hz,Ar-H), 6.32(1H, d, J=16 Hz, CH=C*H*-C=O), 4.98(1H, dd, J=14 Hz, 1.6 Hz), 4.63(1H, dd, J=6.4 Hz, 1.6 Hz), 3.94(3H,s, OCH_3).

Dihydrocholesterol ferulate (33). ^{1}H-NMR (Chloroform-d) δ:7.58(1H,d, J=15.0 Hz, *H*C=CH-C=O), 7.06(1H,dd,J=2.4 Hz,10.8 Hz, Ar-*H*), 7.03(1H, d, J=2.4 Hz, Ar-*H*), 6.90(1H,d, J=10.8 Hz, Ar-*H*), 6.26(1H,d, J=15.0 Hz, CH=C*H*-C=O), 4.82(1H,m, O-C*H*-C), 3.92(3H,s, OCH_3), 2-1.2[30H,m,$(CH_2)_{12}$, $(CH)_6$], 0.91(3H,s,CH_3), 0.89(3H,s,CH_3), 0.88-0.85(9H,2xd, unresolved).

3β–O-Feruloyl-17β-hydroxy-5α-androstane (35). ^{1}H-NMR (Chloroform-d) δ: 7.61(1H,d, J=15.6 Hz, *H*C=CH-C=O), 7.08(1H,dd, J=8.4 Hz, 2, Ar-*H*), 7.03(1H, d, J=1.6 Hz, Ar-*H*), 6.92(1H,d, J=8.4 Hz, Ar-*H*), 6.28(1H,d, J=15.9 Hz, CH=C*H*-C=O), 4.82(1H,sep, J=6.2 Hz O-C*H*-C), 3.92(3H,s, OCH_3), 3.64(2H,t, J=8.4 Hz, C-CH_2),1.54-1.26[$(CH_2)_9$, $(CH)_4$], 0.87(3H,s,CH_3), 0.74(3H,s,CH_3).

Arbutin ferulate (39). ^{1}H-NMR (Chloroform-d) δ: 7.66(1H,d, J=15.9 Hz, *H*C=CH-C=O), 7.2 (1H, s, ferulate Ar-H), 7.1(1H, dd, J=1.24 Hz, 7.94 Hz, ferulate Ar-H), 6.96(2H, d, J=8.89 Hz, arbutin Ar-H), 6.83 (1H, d, J=8.29 Hz, ferulate Ar-H), 6.66(2H, d, J=9 Hz, arbutin Ar-H), 6.42(1H,d, J=15.91 Hz, HC=C*H*-C=O), 4.87(3H,s, OCH_3), 4.73(1H, d, J=5.65 Hz, O-C*H*-O), 4.78(1H, dd, J=1.63 Hz, 11.87 Hz, C-*H*CH-O), 4.58(1H, dd, J=6.59 Hz, 11.89 Hz, C-HC*H*-O)

Arbutin cinnamate (40). ^{1}H-NMR (Chloroform-d) δ: 7.73(1H,d, J=16.08 Hz, *H*C=CH-C=O), 7.62 (2H, m, cinnamate Ar-H), 7.42(3H, m, cinnamate Ar-H), 6.95(2H, d, J=8.98, arbutin Ar-H), 6.66 (2H, d, J=8.81 Hz, arbutin Ar-H), 6.57(1H, d, J=15.96 Hz, HC=C*H*-C=O), 4.75(1H, d, J=6.82 Hz, O-C*H*-O), 4.58(1H, dd, J=1.46 Hz, 11.68, C-*H*CH-O), 4.39(1H, dd, J=6.59 Hz, 11.89, C-HC*H*-O).

3.4 Results and discussion

Regioselective transesterifications catalysed by lipase were achieved in this study. Model acylations of dihydrocholesterol by vinyl acetate were conducted first and the steroid ferulations were based on them. In order to drive the esterification reaction towards synthesis, a large excess of the most readily available acylating agent was used. Thus, in all the transesterifications reactions, an excess of acyl donor was added to the reaction mixture.

3.4.1 Acylation of ferulic acid to produce vinyl ferulate

The chemical synthesis of the vinyl ester of ferulic acid was conducted via a vinyl exchange reaction between vinyl acetate and ferulic acid (Figure 3-4). This was done in order to create a more chemically reactive form of ferulic acid, since the vinyl moiety is a good leaving group. This strategy was employed in order to enhance the subsequent enzymic conversion. The structure of the vinyl ester was confirmed by NMR spectroscopy, and the recovered yield of **26** was 45 %.

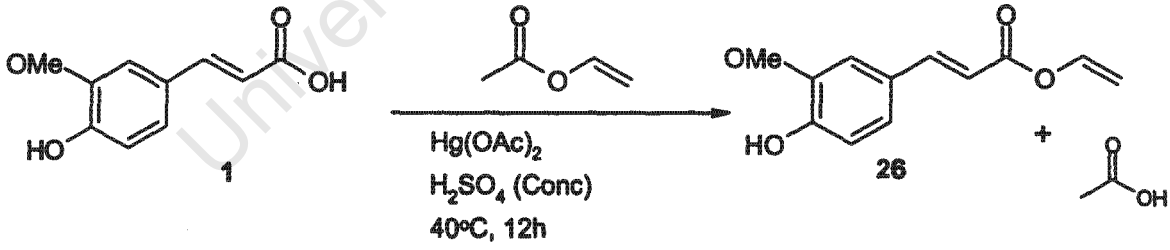


Figure 3-4. Synthesis of vinyl ferulate (26) by a vinyl exchange reaction between vinyl acetate and ferulic acid, as first described by Gao *et al.* (2001).

3.4.1.1 Acetylation of dihydrocholesterol by vinyl acetate

In order to ascertain the ability of *C. antarctica* B lipase to catalyse the acetylation of a steroid by a vinyl ester donor, the acetylation of dihydrocholesterol (**27**) as reported by Bertinotti *et al.* (1994), was attempted, but using tert-butyl-methyl ether as the solvent (Figure 3-5).The progress of the reaction was monitored by TLC using chloroform/methanol (10:0.1) as mobile phase using an authentic sample of 3-*O*-acetyl dihydrocholesterol as a reference. The reaction was successful and ascertained the ability of lipase to acylate dihydrocholesterol. The yield was not calculated as it was a qualitative analysis to establish the reaction under the conditions of the present study.

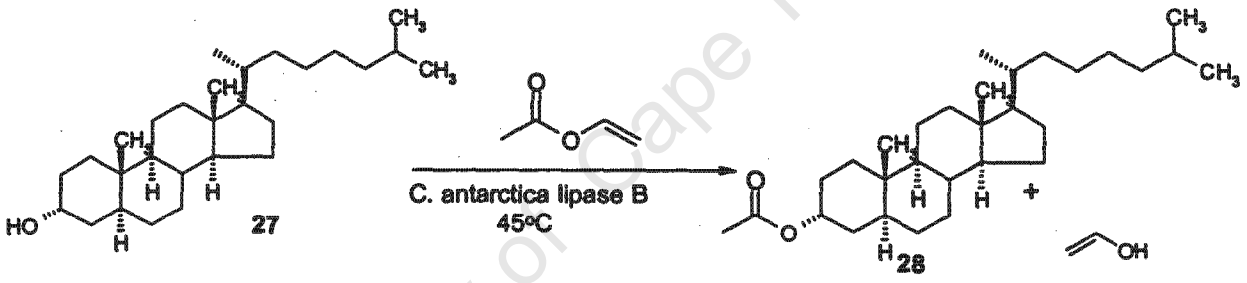


Figure 3-5 . Reaction scheme for the acetylation of dihydrocholesterol catalysed by lipase.

3.4.1.2 Enzymatic transesterifications of vinyl ferulate or trifluoroethyl cinnamate with octanol

To demonstrate the ability of *C. antarctica* lipase B to catalyse transesterification reactions with vinyl ferulate, (**25**) and trifluoroethyl cinnamate (**29**) initially a simple aliphatic alcohol, n-octanol (**30**) was used. This would give an indication of the ability of the lipase to use vinyl ferulate as an acyl donor. Trifluoroethyl cinnamate and vinyl ferulate are good acyl donors, which would increase the reaction yields (Usyatinsky *et al.*, 2003). GC-MS analysis was used to confirm the formation of the expected products (Figures 3-7 and 3-8). The reaction mechanism is shown below in Figure 3-6.

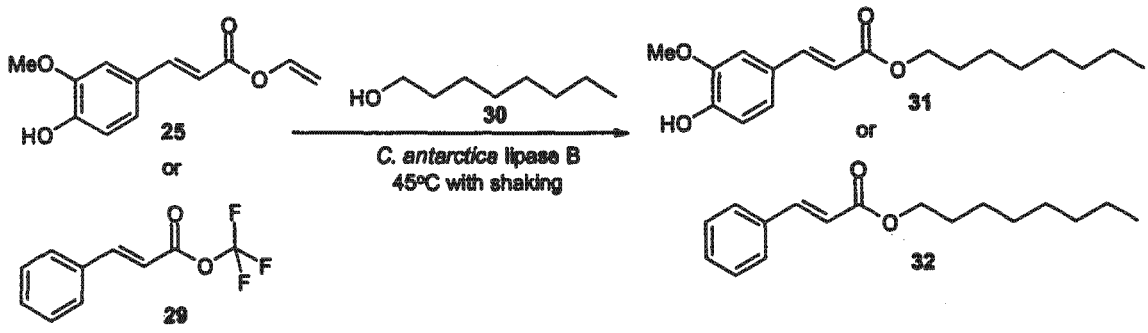


Figure 3-6. Transesterification reactions between trifluoroethyl cinnamate or vinyl ferulate catalysed by *C. antarctica* lipase.

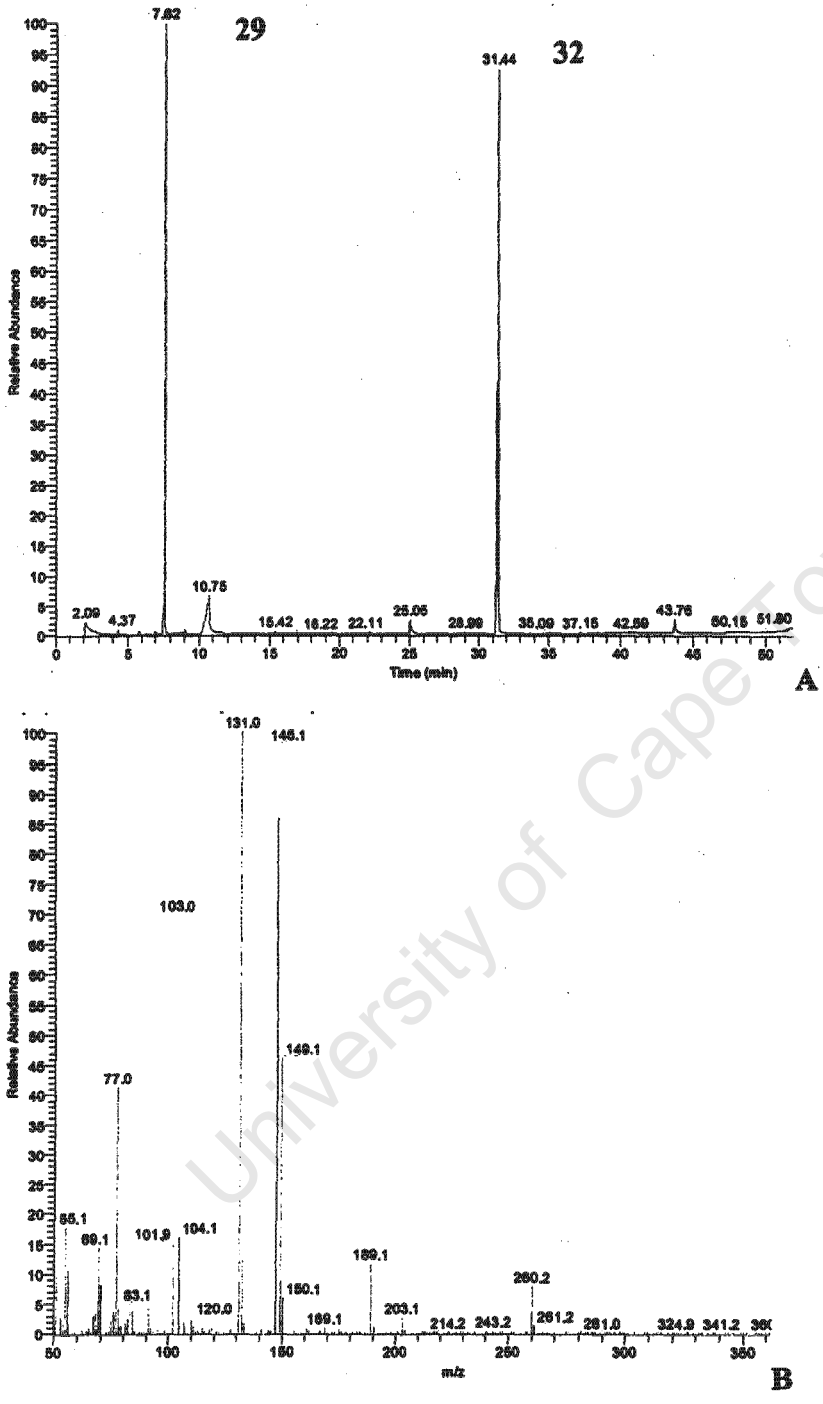


Figure 3-7. GC-MS chromatograms of the products obtained from the lipase-catalysed reaction between trifluoroethyl cinnamate and octanol: A) chromatogram of the reaction mixture, and B) mass spectrum of the second peak (Rt=31.44 min), which is the product, cinnamyl octanoate (32) with m/z = 260.2.

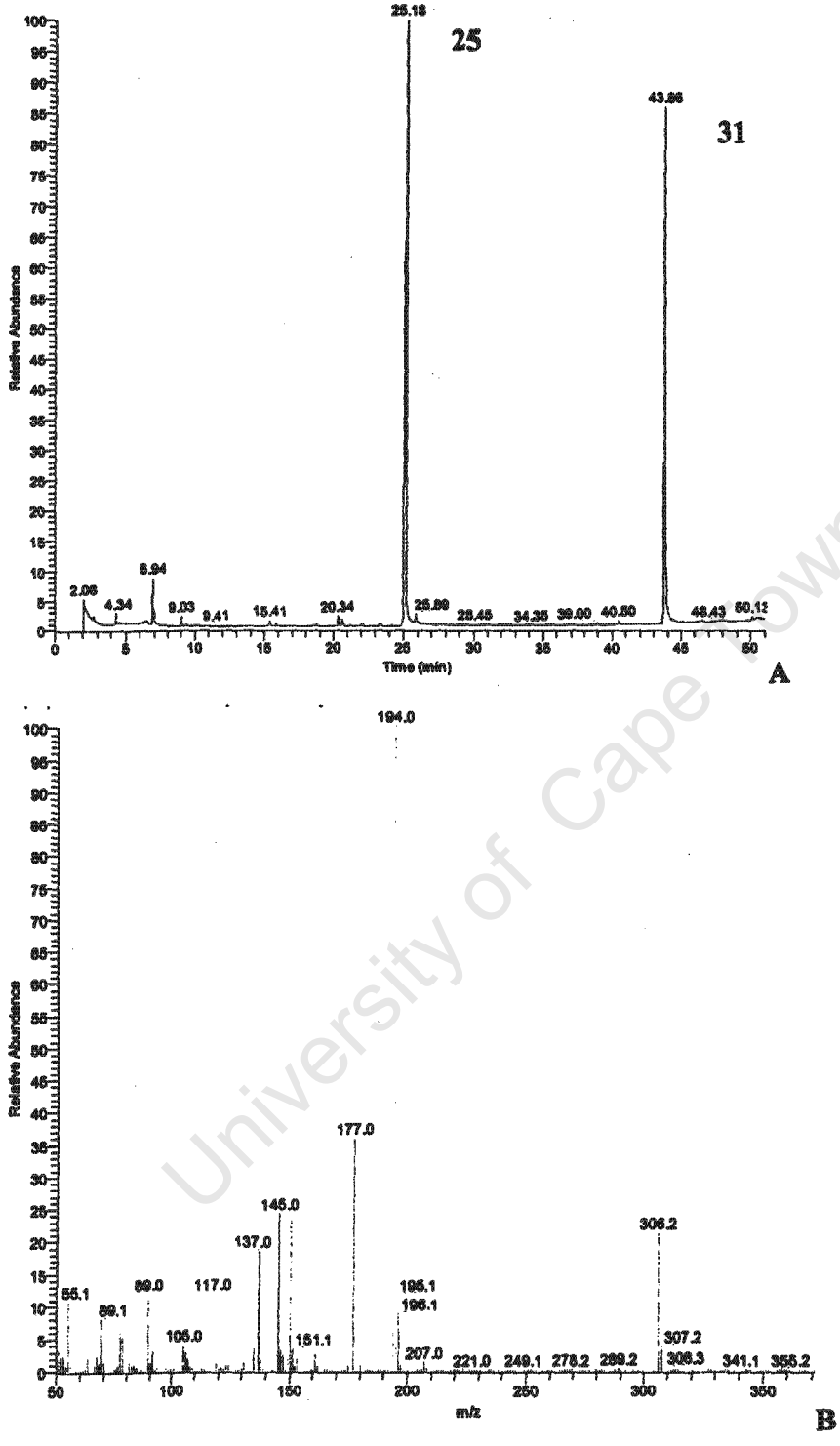
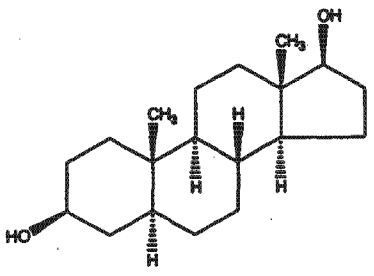


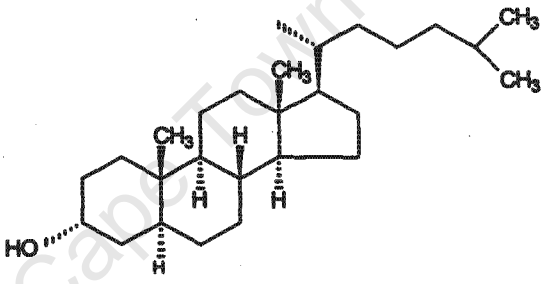
Figure 3-8. GC-MS chromatograms of the products obtained from the lipase-catalysed reaction between vinyl ferulate and octanol: A) chromatogram of the reaction mixture, and B) mass spectrum of the second peak (Rt=43.85 min) which is the product feruloyl octanoate (31) with m/z = 306.2.

3.4.1.3 Biocatalytic synthesis of sterol ferulates

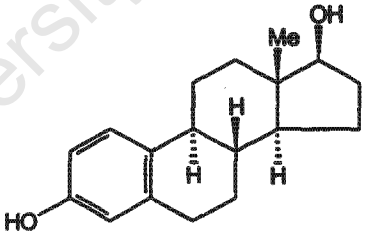
To the best of our knowledge, transesterification reactions between steroid structures and vinyl ferulate have not been reported previously. The reactions were expected to be biocatalytically difficult due to the bulkiness of the two substrates (steroid structures shown in Figure 3-9). This proved to be the case and the transesterification reaction only took place when it was catalysed by the lipase from *C. rugosa* but not in the presence of *C. antarctica* nor *Chromobacterium viscosum*, nor *Pseudomonas* sp.



5α-Androstane-3β,17β-diol
34



Dihydrocholesterol
27



β-Estradiol
37

Figure 3-9. Structures of steroids used for synthesis of steroid derivatives.

The isolated yield for dihydrocholesterol ferulate was 56 % and its structure was confirmed by ^{1}H-NMR analysis (Figure 3-10). Lipases from *Pseudomonas* species are known to be unable to accept bulky substrates and are more selective in their hydrolysis compared to those from *Candida* species, and *C. rugosa* lipases are reported to be able to accept relatively bulky esters (Faber, 1995; Riva *et al.,* 1989). This was confirmed in our reactions, since *C. rugosa* lipase was able to catalyse the transesterifications reaction whereas the *Pseudomonas* species' lipases, were not.

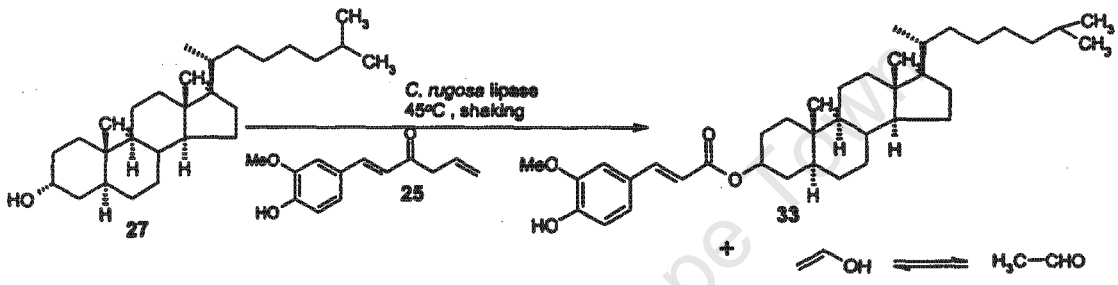


Figure 3-10. Synthesis of dihydrocholesterol ferulate (33) catalysed by the lipase from *C. rugosa*.

Unexpectedly, *C. antarctica* lipase which was previously shown to have activity exclusively on the alcoholic functionalities on steroid A rings (Bettinotti *et al.,* 1994), was also unable to catalyse the transesterification of 5α-androstane-3β,17β-diol (34) with vinyl ferulate. This was attributed to the limited size of the enzyme active site. The reaction took place when catalysed by *C. rugosa* lipase, and the product was isolated with 44 % yield. ^{1}H-NMR analysis was used to confirm that the feruloylation of the diol and to show that it was regioselective towards the 3β-OH, giving 3β–O-Feruloyl-17β-hydroxy-5α-androstane (35).The downfield shift of the 3α-H by the 3β-OH to 4.82 ppm compared to 3.80 ppm in starting material, (appendix B) as a septet with a J value of 6.2 Hz was due to the acylation catalysed by the lipase. Small quantities of a by-product thought to be the diester 3β,17β-feruloyl-5α-androstane (36) were isolated and analysed by NMR. However, due to the small quantities obtained, the structure could not be

confirmed. Figure 3-11 shows the reaction scheme for the production of compounds **35** and **36**. These compounds have not previously been described to the author's best knowledge.

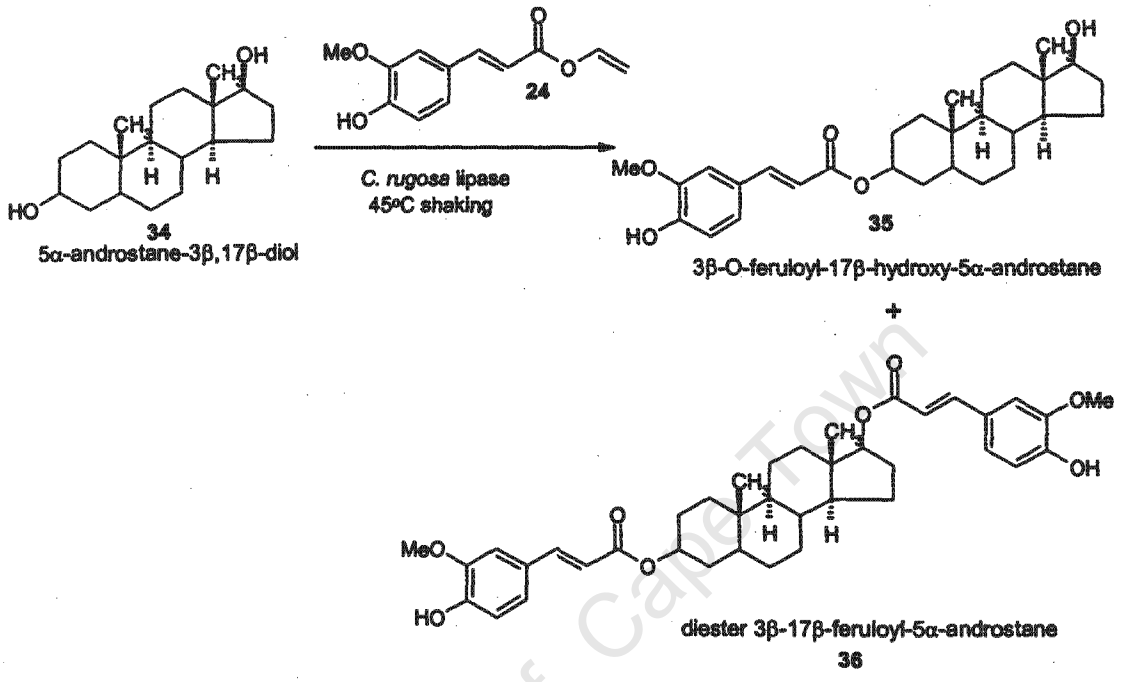


Figure 3-11. Transesterification reaction of 5α-androstane-3β,17β-diol with vinyl ferulate catalysed by *C. rugosa*.

As expected, the lipase-catalysed acylation of β-estradiol was not successful. It has been reported previously that this compound is not a suitable lipase substrate due to the unreactive phenol hydroxyl group and to their inability to acylate 12β-OH (Riva and Klibanov, 1988). However the same researchers showed that subtilisin can acylate in this position and could be used in further studies for this purpose.

3.4.1.4 Enzymatic transesterification of arbutin or trifluoroethyl cinnamate with vinyl ferulate

Arbutin **(38)** is a well-known biologically active compound which acts as a melanogenesis inhibitor (Maeda and Kukuda, 1996). Its depigmenting abilities were demonstrated in melanocytes, and the effect of its derivative with ferulic acid would

potentially possess interesting biological activity. Further studies are planned for testing the compounds effects. The lipase catalysed transesterifications were successfully performed as demonstrated by TLC. Purification of the product was conducted as described in the material and methods section and isolated yields were 59 % for arbutin cinnamate and 50 % for arbutin ferulate. The ^{1}H-NMR spectrum obtained for arbutin cinnamate was identical to that obtained by Nakajima *et al.* (1997). The reaction is illustrated below in Figure 3-12.

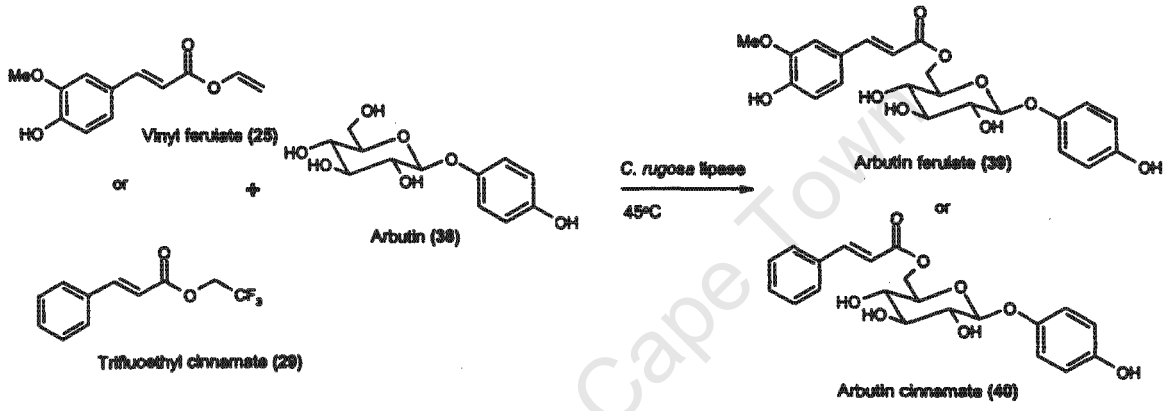


Figure 3-12 . Reaction scheme for lipase catalysed regioselective transesterification to synthesize arbutin ferulate (39) and arbutin cinnamate(40)

3.5 Conclusions

The biocatalytic synthesis of compounds structurally similar to the bio-active cycloartenyl ferulate was successfully achieved using lipase. It was found that lipase from *C. rugosa* possessed the ability to catalyse the reactions while those from *C. antarctica*, *Pseudomonas* sp and *Chromobacterium viscosum* did not The steroid esters of ferulic acid that were synthesised resemble sterol ferulates which are well known nutraceuticals and have pharmaceutical applications. The assessment of the biological activity of the novel compounds as antioxidants and anti-NOX agents is described in the following chapter. This study demonstrates the added versatility of lipases to perform enzymatic transesterifications, broadening the range of their industrial applications.

4 Biological activity of the synthesised products: Screening for antioxidant and anti-NOX activity.

4.1 Introduction

The study of antioxidants has been the subject of increased attention over recent years. As evidence of this, the Medline database had a 340 % increase in papers containing the word antioxidant compared to the 39 % increase in papers in the plant, human and animal fields over the past 10 years (Prior *et al.,* 2005). This is mostly due to the increasing awareness of the health benefits afforded by antioxidants. Numerous different methods of quantifying antioxidant activities have been developed and variations of the different methods also exist. This raises the need for standardised methods of antioxidant determination and quantification. Several factors make this task difficult because antioxidants have widely different chemical and physical properties. In the biological systems in which most antioxidants are relevant, there are four main groups of endogenous and exogenous antioxidants:

- Enzymes *e.g.* superoxide dismutase, catalase
- Large molecules *e.g.* albumin, ceruloplasmin and other proteins
- Small molecules *e.g.* carotenoids, polyphenols, ascorbic acid
- Hormones *e.g.* melatonin, estrogen (Prior *et al.,* 2005)

These antioxidants act either directly or indirectly. Those having an indirect antioxidative role do not interact with free radicals or biomolecule radicals, but they regenerate other oxidised antioxidants (Kirsh and de Groote, 2001).

Adding to the complexity of antioxidant systems are the range of chemically different oxidants and free radicals with different physico-chemical properties e.g. $HO^{\bullet}$, $NO^{\bullet}$, $O_2^{\bullet}$. As a result of this variety, an antioxidant may have several different antioxidant mechanisms *in vivo*. For example, flavonoids have been found to protect neuronal cells from oxidant stress by three distinct mechanisms, namely: by increasing intracellular

GSH and antioxidant enzymes, by directly lowering ROS concentrations, and by preventing Ca^{2+} influx that is ROS dependent (Ishige *et al.,* 2001). An antioxidant may also act by a particular mechanism depending on the reaction system, for example, in different media, or in response to different radicals or oxidants (Prior *et al.,* 2005). Polyphenols, a well-known group of antioxidants, have also been reported to have multiple activities, with the dominant activity being dependent on the medium and the oxidant involved (Prior *et al.,* 2005). Furthermore, their measured antioxidant activity varies depending on the reactants and assay condition used. Thus, in lipid-based media as opposed to aqueous media, or in reactions with different ROS, the antioxidant activity measured may vary widely. As a result, it is important to compare the antioxidant activities of different compounds under identical conditions, and to use multiple assays.

Hydrogen Atom Transfer, (HAT)-based methods of measuring antioxidant activity are based on the ability of an antioxidant to quench free radicals by hydrogen donation. Single Electron Transfer, (SET) methods measure the ability of antioxidants to reduce free radicals by the addition of one electron. These mechanisms were explained in more detail in Chapter 1 (sections 1.4.3.2 and 1.4.3.3).

The objective of the work described in this chapter was to assess the antioxidant activity of ferulic acid derivatives that were synthesised during the course of this study. Several antioxidant assays were used which included both HAT and SET methods. In addition, a novel antioxidant method, based on the inhibition of NADH oxidase (NOX) activity was developed and assessed.

4.1.1 Assays used to measure antioxidant activity

4.1.1.1 The 2,2-Diphenyl-1-picrylhydrazyl, (DPPH$^{\bullet}$) assay

This assay is based on the ability of test compounds to reduce the stable free radical DPPH$^{\bullet}$ (Figure 4-1). The free radical has an absorbance at 515 nm which disappears upon reduction by an antioxidant or a radical, as shown below:

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

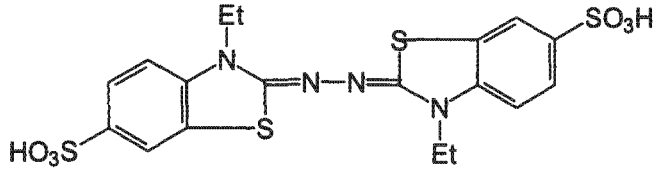
$$DPPH^{\bullet} + R^{\bullet} \longrightarrow DPPH\text{-}R$$

Antioxidant activity can then be determined by monitoring the decrease in absorbance at 515 nm (Brand-Williams *et. al.* 1995). The mechanism of DPPH free radical quenching uses both the HAT and SET mechanisms (Jiminez *et al.*, 2004).

4.1.1.2 Trolox Equivalent Antioxidant Capacity, TEAC assay

The TEAC assay, like the DPPH assay, is an antioxidant capacity assay utilising both HAT and SET mechanisms of quenching free radicals (Jimenez *et al.*, 2004). The assay is based on the scavenging ability of antioxidants reacting with the stable radical anion 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid), ABTS$^{\bullet+}$ (Figure 5-1). Unlike the DPPH assay, the radical chromophore is not commercially available and is generated by reacting ABTS with potassium persulphate. The ABTS method used in this study was as reported by Re *et al.* (1999). Decolourisation of the ABTS$^{\bullet+}$ chromophore due to quenching of the radical by antioxidants, forms the basis of the assay. Antioxidants added to the radical reduce it to ABTS, in direct proportion to their antioxidant activity, concentration and duration of the reaction. The extent of decolourisation, measured at 734 nm and calculated as percentage inhibition of ABTS$^{\bullet+}$, is determined as a function of concentration and time. The reactivity of the test antioxidants was calculated relative

to the reactivity of the synthetic antioxidant trolox, used as a reference. Trolox is a water-soluble vitamin E analogue (Antolovich *et al.*, 2002).



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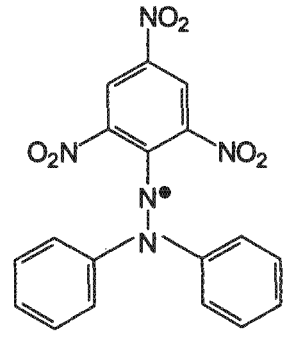


42

Figure 4-1. Structures of free radicals used in antioxidant assays. 41, structure of 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid), $ABTS^{\bullet+}$, 42, structure of 2,2-Diphenyl-1-picrylhydrazyl, $DPPH^{\bullet}$

4.1.1.3 Inhibition of oxidation of low density lipoproteins, LDLs

The early stages of lipid oxidation result in the formation of 1, 4-conjugated dienes which can be detected spectrophotometrically, providing a useful method for the measurement of lipid oxidation (Heinonen *et al.* 1998; Antolovich *et al* ., 2002). The substrates used are polyunsaturated fatty acids and oxidation can be initiated by copper or iron ions, heat or chemical reagents. Conjugated dienes are formed from the hydrogen abstraction of CH_2 groups on fatty acids, and the absorbance of the dienes is measured spectroscopically (Antolovich *et al.*, 2002).

The well-supported oxidation hypothesis of atherogenesis proposes that the oxidative modification of LDL's results in the formation of artheromas, which are the main cause of coronary heart disease (Steinberg *et al.*, 1989; Steinberg, 1988). LDLs as their name suggests are composed of lipids and proteins. They are the lipid carriers in the blood and consist of an oil droplet (containing cholesterol esters) surrounded by a layer of phospholipids and protein (Mathews and van Holde, 1990). Human serum lipid extracts were found to contain diene conjugated material and it was suggested that this reflected

oxidation *in vivo* (Dormandy and Wickens, 1987). Antioxidants can reduce the extent of LDL oxidation caused by ROS (Meyer *et. al.* 1997). As a result, the inhibition or prevention of the oxidation of the lipid component of LDL, is a useful antioxidant assay. This is because it is more representative of *in vivo* conditions as it is based on the oxidation of LDL which is found *in vivo* and not on reactions of non-natural compounds.

The ability of the biocatalytically synthesized compounds produced in this study to inhibit copper (II)-mediated LDL oxidation was investigated. The oxidation was measured by monitoring the formation of conjugated dienes spectrometrically at 234 nm (Nardini *et al.,* 1995).

4.1.1.4 Inhibition of NADH oxidase activity by antioxidants

NAD^+ and NADH are well known as co-enzymes. They are powerful redox agents and both are essential in many biosynthetic processes, in which they are continually being used and recycled in cells. Upon its reduction, NADH is released into the cytosol to be reoxidised by other molecules. This NADH can then reduce further biomolecules (Mathews and van Holde 1990).

NADH and its reduced form, NAD^+, both play important roles in cellular metabolic processes and are found in various proportions in cells. Glycolysis is the central metabolic pathway which generates energy and other metabolic intermediates from glucose (van Holde 1990). NAD^+ is used as a cofactor by 2 enzymes in the pathway, producing NADH. This NADH is reoxidised in the electron transport chain, maintaining a homeostasis in cells. NADH concentration have been found to oscillate in the cell cytosol during glycolysis (Mathews and van Holde, 1990). NADH is therefore needed in glycolysis and it's reduced form NAD^+, is needed in the electron transport chain, which takes place in mitochondrial inner membranes (van Holde, 1990).

The cellular NADH/NAD+ ratio has also been found to change due to conditions of oxidative stress, via the upregulation of enzymes involved in their recycle, such as

NADH oxidase (NOX). Oxidative stress in lactic-acid producing bacteria has been noted to be reduced by NADH oxidases (Bolotin *et al.*, 2001; De Angelis and Gobbetti 2004). Various types of NOX enzymes are thought to protect the cells from oxidative stress arising during glycolysis, and NOX proteins have been found to decrease O_2 stress under aerobic metabolism (Gibson *et al.* 2000, Marty-Teysset *et al.* 2000). NADH oxidases are involved in the oxidation of NADH to NAD^+. The reaction involves a two-electron reduction of molecular oxygen to H_2O_2 or, in the case of water forming- NOX, a four electron reduction :

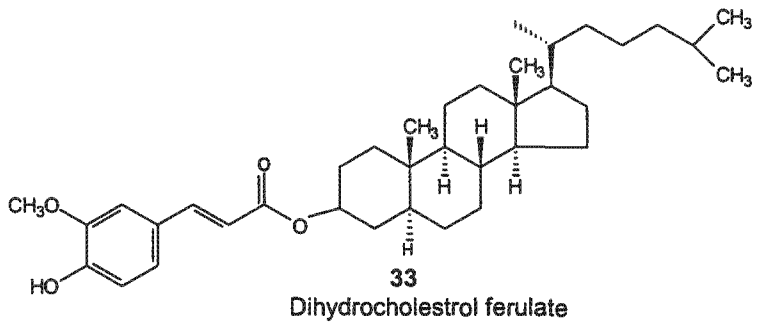
$$\mathbf{2NADH} + \mathbf{O_2} \xrightarrow{\text{NOX}} \mathbf{2NAD^+} + \mathbf{2H_2O}$$

They are commonly found in evolutionarily divergent organisms, and both the water- and hydrogen peroxide- forming NOX are found in many bacteria (Riebel *et al.,* 2002, Hummel and Riebel, 2003).

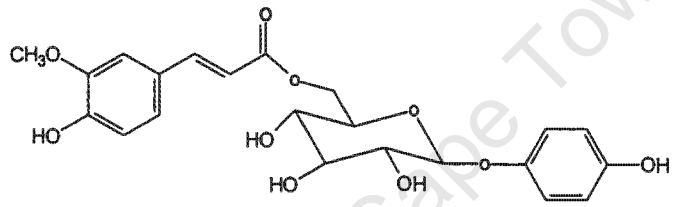
Cellular reduction and oxidation systems have been shown to affect cell proliferation and differentiation (Crane *et al.,* 1990). Membrane redox systems have been shown to have a direct link with growth of tumour cells, which are cells undergoing uncontrolled cell growth (Chueh, 2000). Morre *et al.* (1995), found NOX activity to be elevated in transformed cells compared to corresponding non-transformed cells. Antioxidants such as retinoic acid, 1,25-dihydroxy-vitamin D_3, were found to inhibit NOX activity correlated to the growth of HeLa cells (a cancer cell line) by Morre *et al.* (1992). Thus, cell growth has been found to be controlled by redox reactions occurring in the membrane. NOX activation stimulates cell growth and inhibition of membrane redox activity results in inhibition of cell proliferation (Chueh 2000 and Morre, 1998.)

In this study, based on these findings, a hypothesis that NOX activity could be inhibited by antioxidants was formulated. The inhibition of NOX activity by antioxidants would inhibit cancer cell line proliferation, and antioxidants with anti-NOX activity may have anticancer activity. In order to test the hypothesis, an NADH oxidase was obtained that would be used to measure anti-NOX activity *in vitro*. The aim was to develop an assay

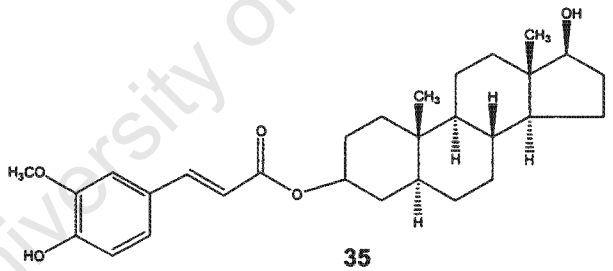
that could be used to screen antioxidants that possess anti-NOX activity that could potentially have applications as therapeutic drug precursors. The antioxidant compounds (Figure 4-2) produced biocatalytically in this present study were then tested for anti-NOX activity.



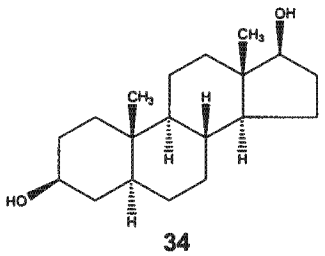
33
Dihydrocholestrol ferulate



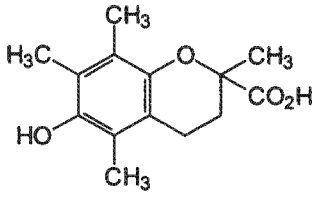
39
Arbutin ferulate



35
3β-O-Feruloyl-17β-hydroxy-5α-androstane



34
5α Androstane 3β,17β diol



Trolox

Figure 4-2. Structure of compounds tested for antioxidant activity.

4.2 Materials and methods

4.2.1 Materials

All solvents and salts were obtained from Merck South Africa. ABTS was purchased from Roche chemicals, Trolox, and DPPH were bought from Sigma Chemicals. NADH oxidase from *Lactobacillus sanfrancisnensis* was a kind gift from Prof. Andreas Bommarius, Georgia Institute of Technology, USA. Phenolic compounds, resveratrol, caffeic acid, ascorbic acid, ferulic acid, dihydrocholesterol, 5α-androstane-3β,17β-diol, arbutin were obtained from Sigma Chemicals. The synthesised products were synthesised as described in Chapter 4. All spectroscopic measurements were carried out using an α-Helios Helicam spectrophotometer at room temperature except where otherwise stated.

4.2.2 DPPH$^{\bullet}$ antioxidant assay

The method used was as described by Bondet *et al.* (1997). A DPPH$^{\bullet}$ standard curve was constructed in order to determine accurate DPPH$^{\bullet}$ quantities. (The linear regression obtained was y = 9973.8x - 0.0091, where y was the absorbance at 515 nm, and x the molarity of DPPH$^{\bullet}$). 3.5 mL of approximately 6 x10^{-5} M methanolic DPPH$^{\bullet}$ (the exact amount was calculated from the standard curve) was reacted with 0.5 mL samples (7.5-15 μM final concentration) of the antioxidant to be tested. Absorbance was monitored until the reaction reached steady state.

4.2.3 TEAC assay

The method used was as described by Re *et al.*, (1999). ABTS$^{\cdot +}$ was generated by reacting 7 mM ABTS and 2.45 mM potassium persulphate in water. The solution was stored in the dark for 12-16 hours. The free radical solution was then diluted with 1 x

phosphate buffered saline, (PBS) pH 7.4 (see appendix), to an absorbance of 0.70 ± 0.02. 10 µL volumes of the tested antioxidants (1.2-0.6 µM final concentration) dissolved in ethanol were added to 990 µL of the diluted $ABTS^{\cdot+}$ solution. The change in absorbance at 734 nm was measured. All tests were carried out and measured at 30 ± 2°C, in triplicate. The Trolox equivalent antioxidant activity, (TEAC), of the antioxidant is defined as the "concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation at 734 nm as 1 mM of Trolox, at specific time points"(Re *et al.*, 1999) calculated relative to the decrease in absorbance of Trolox at specific time point. This was calculated after 6 minutes for this study.

4.2.4 LDL oxidation

Oxidation of low density lipoprotein (LDL) was performed according to the method developed by Nardini *et al.* (1995). The LDL was dialysed in a 200 fold volume of PBS pH 7.4 at 4°C in the dark for 18h. 100 µg/mL LDL (determined by Bradfords method of protein determination) was then oxidised using 5 µM $CuCl_2$ for 4 hours at 37°C in the presence or absence of 50 µM test antioxidant. Conjugated diene formation was measured spectroscopically at 234 nm. Samples were assayed in duplicates

4.2.5 NADH Oxidase activity assays

(This section of work was done in collaboration with Miss Marie Blanche Ting.)
In a 3 mL cuvette, 0.3 mL of 1M triethanolamine, (TEA) was added with 0.3 mL 2 mM NADH in water and 0.03 mL of NADH oxidase, the reaction mixture was then made up to 3 mL with double distilled water. The reaction was carried out at 30°C. The disappearance of NADH was measured at 340 nm. The blank consisted of 0.3 mL TEA with water making up the rest of the 3 mL volume. The enzyme reaction was followed over time and the activity was calculated using an extinction coefficient ε, of NADH of

6.22 L/(mol cm) (Riebel *et al.*, 2002). The disappearance of NADH is inversely proportional to enzyme activity. Samples were run in duplicate or triplicate.

4.2.5.1 Effects of organic solvent on the NADH oxidase, NOX.

0.03 mL and 0.1 mL of ethyl acetate and methanol were added to the reaction mixture before NOX and assayed as described in section 4.2.5. The experiments were conducted in duplicate.

4.2.5.2 Experimental analysis of absorbance changes due to interaction of NADH and test compounds.

300 μL volumes of 100 mM test compounds, resveratrol, caffeic acid, ferulic acid, vinyl ferulate, naringin, catechin, eugenol, *p*-arbutin, catechol and ascorbic acid, dissolved in methanol, were added to 0.3 mL of NADH in water (2 mM) and 0.3 mL of TEA (1 M). Each reaction mixture was made up to 3 mL with double distilled water and the absorbance read at 340 nm, to determine the increase over time. Samples were run in duplicate or triplicate.

4.2.5.3 NOX inhibition studies: addition of phenolic compounds prior to addition of enzyme

The investigation of test compounds, namely: resveratrol, caffeic acid, ferulic acid, catechin, eugenol, *p*-arbutin, catechol and ascorbic acid, on enzyme activity, was carried out with the compounds added to the reaction mixture before the enzyme, and the effect observed. Solutions with final concentrations of 0.2 mM and 0.02 mM concentration of resveratrol, and 1 mM of caffeic acid and ferulic acid, dissolved in methanol, were added to 0.3 mL of NADH (2 mM) and 0.3 mL of TEA (1 M). This reaction mixture was made up to 3 mL with double distilled water and then 0.03 mL of enzyme was added.

4.2.5.4 NOX inhibition studies: addition of inhibitor after addition of enzyme

The effect of adding putative inhibitor compounds after 40 seconds of reaction between NOX and NADH was investigated. Test compound concentrations of 0.1x, 0.2x and 1x the concentration of NADH were investigated. The enzyme activity after the addition of the inhibitor was calculated using an extinction coefficient ε, of NADH of 6.22 L/(mol cm) (Riebel *et al.,* 2002), and compared to the corresponding enzyme activity in the absence of putative inhibitor.

4.3 Results and discussion

There is a need for a multiple assay approach when testing for antioxidant activity (Frankel and Meyer, 2000). This is due to the variation in reaction mechanisms of antioxidants and their sensitivity to their environment. "Colloidal properties of substrates, conditions and stages of oxidation and localisation of antioxidants in different phases" are all important factors to be considered (Frankel and Meyer, 2000). When testing antioxidant activity *in vitro,* system compositions as close as possible to *in vivo* systems become important because antioxidants perform their functions *in vivo*. These conditions are not easy to mimic, and hence there is benefit in a multiple assay approach using different media and substrates, specifically using both hydrophobic and hydrophilic systems, as is the case *in vivo*. In this study, three different antioxidant assays were used in order to obtain a clearer indication of the antioxidant activities of the compounds synthesised.

Physiologically, the concentrations of antioxidants are low, in the micromolar range (Valko *et al.,* 2006; Orallo *et al.,* 2002) while some antioxidants in blood plasma are in the nanomolar range (Zhu *et al.,* 1999). Therefore based on this, micromolar quantities of putative antioxidants were used in order to obtain results more pertinent to physiological conditions.

4.3.1 DPPH$^{\bullet}$ antioxidant assay

Brand-Williams and co-workers (1995), first developed the DPPH assay for determination of antioxidant activity. The DPPH radical, DPPH$^{\bullet}$, has an absorption band at 515 nm, which disappears upon reduction by an antioxidant. Hence, antioxidant activity can be determined by monitoring the decrease in absorbance. In these assays, the reaction between the test antioxidant and DPPH$^{\bullet}$ is allowed to continue until it reaches a

steady state and the decrease in absorbance is correlated to a DPPH$^{\bullet}$ dose-response calibration curve. This allows calculation of the amount of DPPH$^{\bullet}$ remaining as a percentage of the initial DPPH$^{\bullet}$ as shown below:

$$\% \text{ DPPH}^{\bullet}_{\text{Remaining}} = 100 \text{ x } [\text{DPPH}^{\bullet}_{\text{Remaining}}]/ [\text{DPPH}^{\bullet}_{\text{initial}}]$$

The amount of the radical quenched at steady state is dependent on the antioxidant ability and concentration of antioxidant (Brand-Williams *et al.*, 1995). The time taken to reach steady state is also a function of antioxidant ability, this being the inherent capability of a compound to withstand oxidation, conferred by its physico-chemical properties.

The work described in this section of the thesis investigated the anti-free radical activity against the free radical DPPH$^{\bullet}$ of ferulic acid and the biocatalytically synthesised ferulic acid esters. The antioxidants were tested at the same molar concentration and the DPPH quenched calculated as

$$\% \text{ DPPH}^{\bullet}_{\text{Quenched}} = 100 - \% \text{ DPPH}^{\bullet}_{\text{Remaining}}$$

The results obtained are shown in Table 4-1 below.

Table 4-1. Antioxidant activities of compounds tested using the DPPH assay

Compound (15 μM final concentration)	Compound name	% DPPH$^{\bullet}$ remaining after 2.5 hours
1	Ferulic acid	59.89
39	Arbutin Ferulate	88.99
33	Dihydrocholesterol ferulate	64.85
34	5α,-androstane 3β,17β diol	93.69
1,35	1:4 (ferulic acid: 3β–O-Feruloyl-17β-hydroxy-5α-androstane and ferulic acid mixture	37.32
35	3β–O-Feruloyl-17β-hydroxy-5α-androstane	76.97

Antioxidant and DPPH$^{\bullet}$ reaction mechanisms depend on the structural conformation of the antioxidant (Brand-Williams *et al.,* 1995). Brand-Williams *et al.* (1995) reported three types of kinetic behaviour from 20 different antioxidants under identical reaction conditions. These are listed below:

- Rapid kinetic behaviour. The reaction takes place rapidly and steady state of the reaction with DPPH$^{\bullet}$ is reached within one minute of reaction time. Examples included ascorbic acid and isoeugenol (Brand-Williams *et al.,* 1995).
- Intermediate behaviour. The reaction reaches steady state after about 5- 10 minutes as observed for δ-tocopherol. Antioxidant compounds with rapid or intermediate reaction kinetics with DPPH$^{\bullet}$, were found to have stoichiometries related to the number of reactive hydroxyl groups in their structures. For example, one isoeugenol molecule, which has one hydroxyl group, reduces one DPPH$^{\bullet}$ molecule while ascorbic acid, which contains two, reduces two molecules of DPPH$^{\bullet}$ (Brand-Williams *et al.,* 1995).
- Slow behaviour. The kinetics of the antioxidant reaction with DPPH$^{\bullet}$ forms a hyperbolic curve with steady state only being reached in 1 to 6 hours. Researchers have identified ferulic acid, eugenol, caffeic acid and other phenolic compounds as belonging to this group and the kinetics of the reactions of the compounds in this group were found to be complex. The kinetics could not be correlated to the stoichiometries only (Brand-Williams *et al.,* 1995), and hence their antioxidant efficiencies could not be related to their stoichiometries alone (Bondet *et al.,* 1997). Some very well known antioxidant compounds belong to this group such as BHT, which reduces 3 moles of DPPH$^{\bullet}$ per molecule. However, BHT only has one hydroxyl group available for the reaction, and the reaction only reaches steady state after a long time (5h at 20°C). As a result it may be concluded that compounds with slower reaction kinetics that take longer to reach steady state in effect are more beneficial as they quench more free radicals.

All but two of the compounds synthesised biocatalytically, when assessed for antioxidant activity using the DPPH$^{\bullet}$ antioxidant assay, were found to fall into the last group. This was based on the hyperbolic kinetic curves obtained as shown in Figure 4-3, for these compounds. The parent compound, ferulic acid, was previously reported, and also observed in this study, to belong to this group (Brand-Williams *et al.*, 1995). Slow kinetic behaviour was observed and steady state was reached after 20 minutes. The ranking order of the antioxidants is shown in Table 4-2. Figure 4-3 shows the decrease in absorbance at 515 nm of the reactions due to the quenching of the DPPH$^{\bullet}$ by the antioxidants. The highest concentration of DPPH$^{\bullet}$ quenched by 15 μM antioxidant was by the 1:4 ferulic acid: 3β–O-Feruloyl-17β-hydroxy-5α-androstane, and the least by arbutin ferulate (Figures 4-3 and 4-4). The mixture of ferulic acid and compound 35 was obtained after a partial purification of compound 35, of which NMR analysis showed the composition (shown in appendix 7.3)

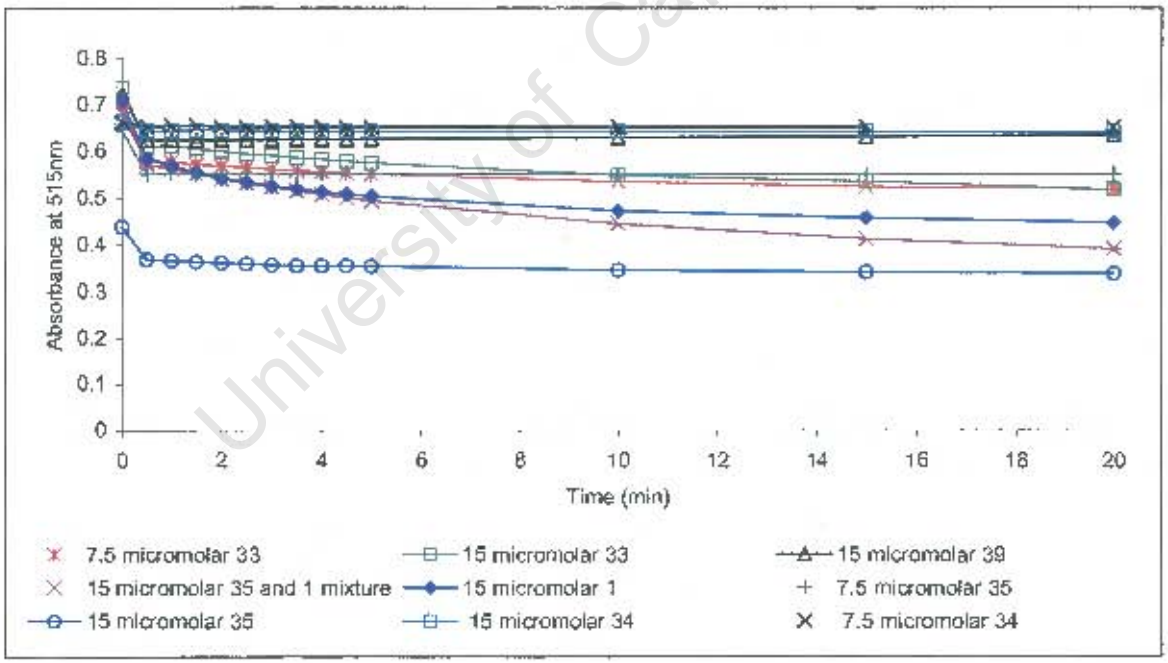


Figure 4-3. Decrease in absorbance at 515 nm of DPPH$^{\bullet}$ free radical chromophore due to quenching by various antioxidants.

Table 4-2. The antioxidant activity of 15μM antioxidant products

Compound	Name	% DPPH$^{\bullet}$ remaining after 20 minutes	Time to reach steady state	% DPPH$^{\bullet}$ at steady state
1	Ferulic acid	62.92	50 minutes	60.23
33	Dihydrocholesterol ferulate	69.90	80 minutes	64.99
34	5α,-androstane 3β,17β diol	93.69	4.5 minutes	93.69
35	3β–O-Feruloyl-17β-hydroxy-5α-androstane	76.97	0.5 minutes	76.97
39	Arbutin ferulate	87.74	0.5 minutes	88.99*
1:4 **1,35** mixture	1:4 Ferulic acid:3β–O-Feruloyl-17β-hydroxy-5α-androstane	56.73	Not reached after 150 minutes	37.32

* there was a small increase in absorbance between 5 and 20 min.

The reactions of DPPH$^{\bullet}$ with ferulic acid esters produced biocatalytically continued longer than the antioxidant starting compounds, ferulic acid and 5α,-androstane 3β,17β diol. Furthermore, the reaction between the mixture of ferulic acid and 3β–O-feruloyl-17β-hydroxy-5α-androstane with DPPH$^{\bullet}$ had not reached steady state after 160 minutes, compared to steady state for ferulic acid being reached after 50 minutes as shown in Figure 4-3 and Table 4-2. It is proposed that slower kinetics are more beneficial *in vivo* and antioxidants that possesses activity over a longer period of time would provide sustained antioxidative protection against the variety of cellular ROS.

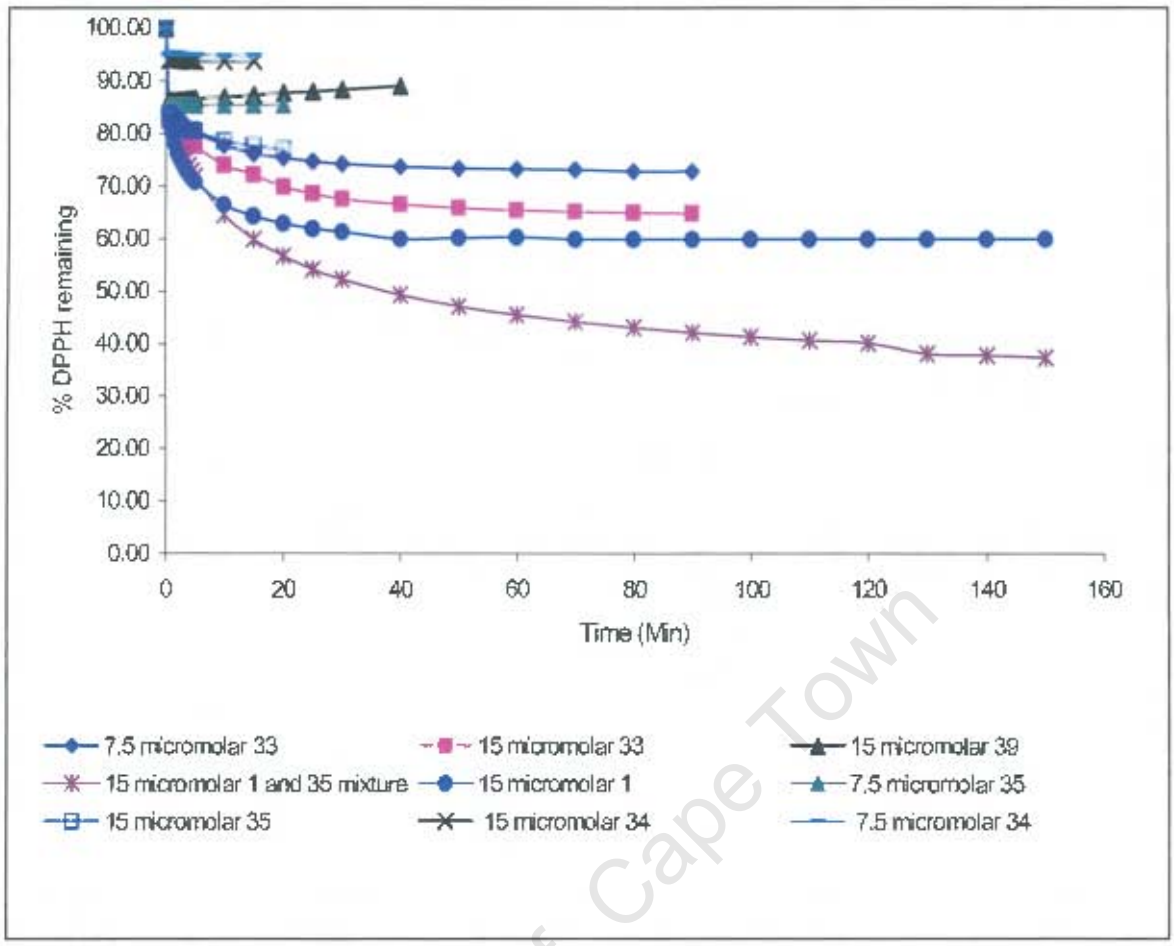


Figure 4-4. Percentage DPPH$^{\cdot}$ remaining over time after reaction with various compounds

The results obtained with the 1:4 ferulic acid: 3β–O-feruloyl-17β-hydroxy-5α-androstane mixture suggests a possible synergistic antioxidant effect. After 2.5h the ferulic acid alone had stoppcd reacting and had quenched 39.77 % of the initial DPPH$^{\cdot}$ radical whereas the mixture quenched 62.68 % after the same period of time. 3β–O-Feruloyl-17β-hydroxy-5α-androstane alone quenched 23 % of the initial DPPH$^{\cdot}$ after 20 min, compared with 43.3 % quenched after the same period of time by the mixture. (The reaction between the DPPH$^{\cdot}$ and pure 3β–O-feruloyl-17β-hydroxy-5α-androstane had reached steady state at 20 min. but its mixture with ferulic acid had not reached steady state even after 2.5h). Only 30.98 % DPPH (Table 4-3) would have been quenched if the

amount quenched was due to an additive effect with no synergistic effect, however, double that amount of DPPH free radical was quenched by the mixture.

Table 4-3. Results of DPPH free radical quenched by ferulic acid (1) and 3β–O-feruloyl-17β-hydroxy-5α-androstane (35).

Compound	Amount DPPH quenched after 2.5h
15μM **35**	39.77 %
1 in 1:4 15μM **1,35** mixture	7.95 %[2]
15μM **35** alone	23.03 %
Calculated DPPH to be quenched by additive effect.	30.98 %[3]
Actual DPPH quenched	**62.68 %**

Similar synergistic effects have been observed previously (Packer *et al.*, 1995; Lu and Liu, 2002), where it was reported that antioxidants can interact with each other to give antioxidant effects amounting to more than just an additive effect. Lu and Liu, (2002) found that certain hydroxycinnamic acids (HCA), including ferulic acid, recycled the antioxidant lipoic acid (LipSS) from its free radical $LipSS^{+\bullet}$ by donating electrons to it, thereby increasing its antioxidant capacity (Figure 4-5).

$$\mathbf{LipSS^{+\bullet} + HCA \longrightarrow LipSS + HCA^{\bullet} \xrightarrow{pH>5} HCA(-H)^{\bullet}}$$

Hydroxycinnamic acid phenoxyl radical

In the present study, the antiradical effectiveness of ferulic acid was markedly increased by the presence of 3β–O-Feruloyl-17β-hydroxy-5α-androstane. The ester itself exhibited stronger free radical quenching ability than ferulic acid (its precursor). A synergistic relationship between ferulic acid and its ester, similar to that between lipoic acid and

[2] DPPH quenched by **1** in 1:4 **1:35** mixture = 0.2 x DPPH quenched by **1**
[3] Calculated DPPH quenched by 1:4 **1:35** mixture from additive effect=DPPH quenched by **1**+ DPPH quenched by **35**

ferulic acid as demonstrated by Lu and Liu (2002), is possible. The nature of the electron donating partnership between lipoic acid and ferulic acid is illustrated in Figure 4-4, and it is proposed that a similar interaction occurs between ferulic acid and its ester.

This hypothesis could be validated by the measurement of the redox potentials of the product and comparing it to that of ferulic acid, because compounds with a lower redox potential can donate electrons to those with higher ones, as this is thermodynamically favourable. However, the assessment of redox potentials did not fall into the scope of this study.

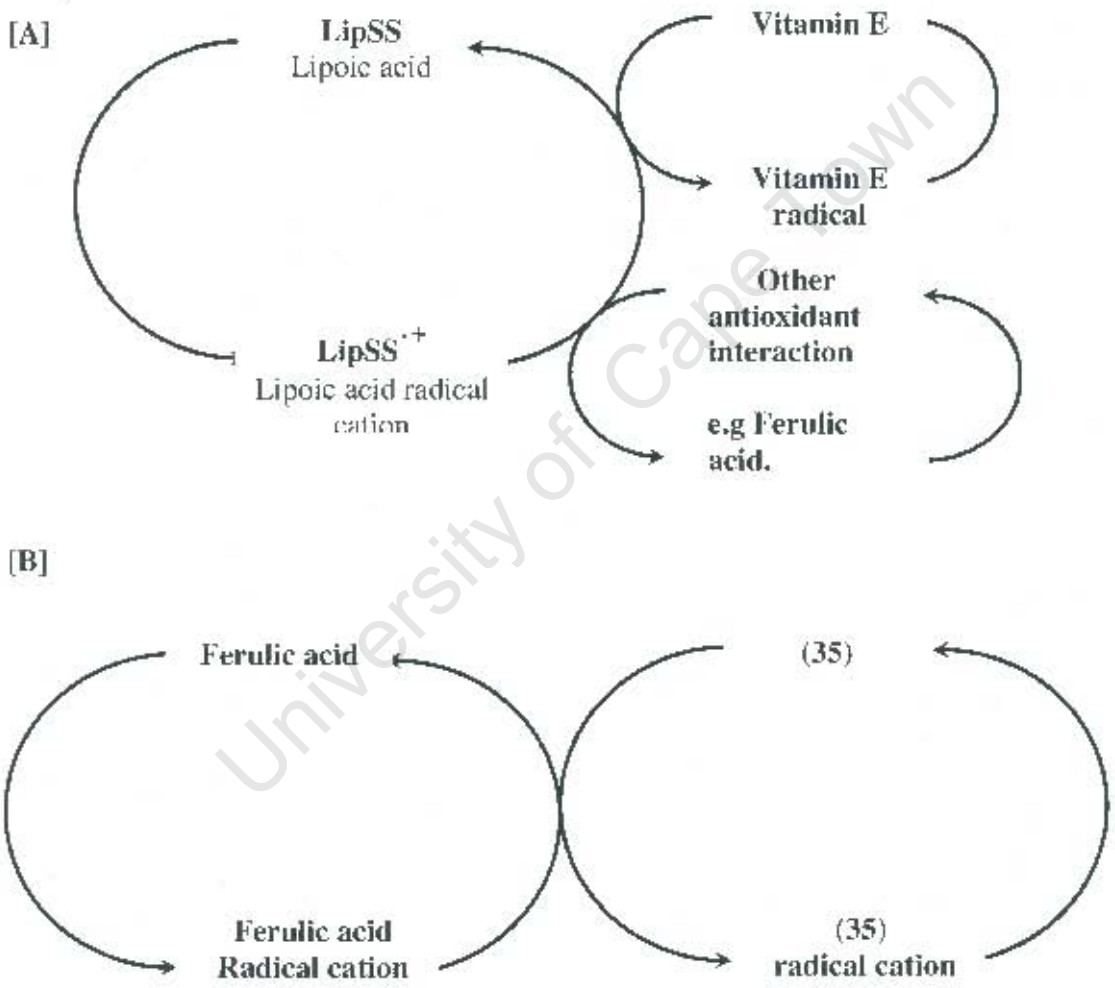


Figure 4-5. Model of proposed synergy of [A], lipoic acid with exogenous ferulic acid and endogenous vitamin E antioxidants (Lu and Liu, 2002) and [B], the proposed synergy between ferulic acid with product 35.

Synergistic interactions of antioxidants play an important role in the prevention of oxidative damage in cells. This is observed for example in disease and ageing (Packer *et al.*, 1995), where there is interaction between endogenous and exogenous antioxidants in cells, which is dependent on their respective redox potentials (Buettener, 1993). The novel products synthesised in this study apparently show such interactions and thus may have therapeutic significance. Antioxidant synergism increases antioxidant effectiveness as lower antioxidant concentration can be used (Moure *et al.*, 2001).

Compared with ferulic acid and the 1:4 ferulic acid:compound **35** mixture, arbutin ferulate **39**, and 3β–O-feruloyl-17β-hydroxy-5α-androstane, **35** showed different kinetics in reactions with $DPPH^{\bullet}$. Both products, unlike the others tested (for example **33** dihydrocholesterol ferulate), reached steady state within one minute reaction time at the reaction conditions used in this study (Figure 5-3 and Table 5-2). The reaction kinetics did not form a hyperbolic curve as the others did and therefore it was concluded that they possessed a different kinetic behaviour. According to Brand-William *et al.* (1995), this behaviour may be classified as rapid kinetic behaviour, since the reactions of the antioxidants with $DPPH^{\bullet}$ reach steady state within a minute and do not form a hyperbolic curve. The compounds **39** and **35** also possessed the lowest anti- $DPPH^{\bullet}$ free radical quenching capabilities (11 and 23 % $DPPH^{\bullet}$ quenched at steady state respectively). There was a slight increase over time in the absorbance of the reaction mixture of $DPPH^{\bullet}$ and **39** between 5 and 10 minutes, which indicted an increase of $DPPH^{\bullet}$ free radical (Figure 4-3). This possible pro-oxidant behaviour was minimal (1.25 %) and was disregarded.

4.3.1.1 Relationship between structures of compounds tested with antioxidant activity.

It is well understood that structural conformation of compounds has an effect on the reaction between $DPPH^{\bullet}$ and the compounds (Brand-Williams *et al.*, 1995) and this effect was observed with the compounds tested in this study. Arbutin ferulate had the least anti-

DPPH$^{\bullet}$ activity (11 % DPPH$^{\bullet}$ quenched) and this may be due to the sugar moiety in its structure. The steroid esters of ferulic acid had better activity, with the dihydrocholesterol ester, **33**, possessing better activity than the androstane ester of ferulic acid, **35**. (See Figure 4-5 for structures of compounds). This difference may be due to the alkyl branch on product **35**, which **33** does not possess. Further investigation would determine the effect of the alkyl group on anti- DPPH$^{\bullet}$ activity but the number of compounds used for this study were insufficient to comment on the effects.

4.3.2 Antioxidant capacity of ferulic acid by laccase reaction products

In view of the potential for the ferulic acid-laccase products to have higher antioxidant capacity than that of ferulic acid itself, a standard antioxidant assay was first used in a preliminary study, to compare the free-radical quenching capacity of the product with that of ferulic acid. Thus, the DPPH assay was used to assess the products obtained by reacting ferulic acid with laccase.

The product mixture from the aqueous reaction of 10 mM ferulic acid by laccase was used for the analysis. The products were previously found to be ferulic acid polymers (Chapter 2). Phenolic polymers have in some cases been found to be more effective antioxidants than the monomers (Ting, 2004). The assays indicated that on the basis of equivalent amounts of converted and unconverted ferulic acid, the antioxidant activity of the product is approximately twice that of ferulic acid. Results are shown in Figure 4-6.

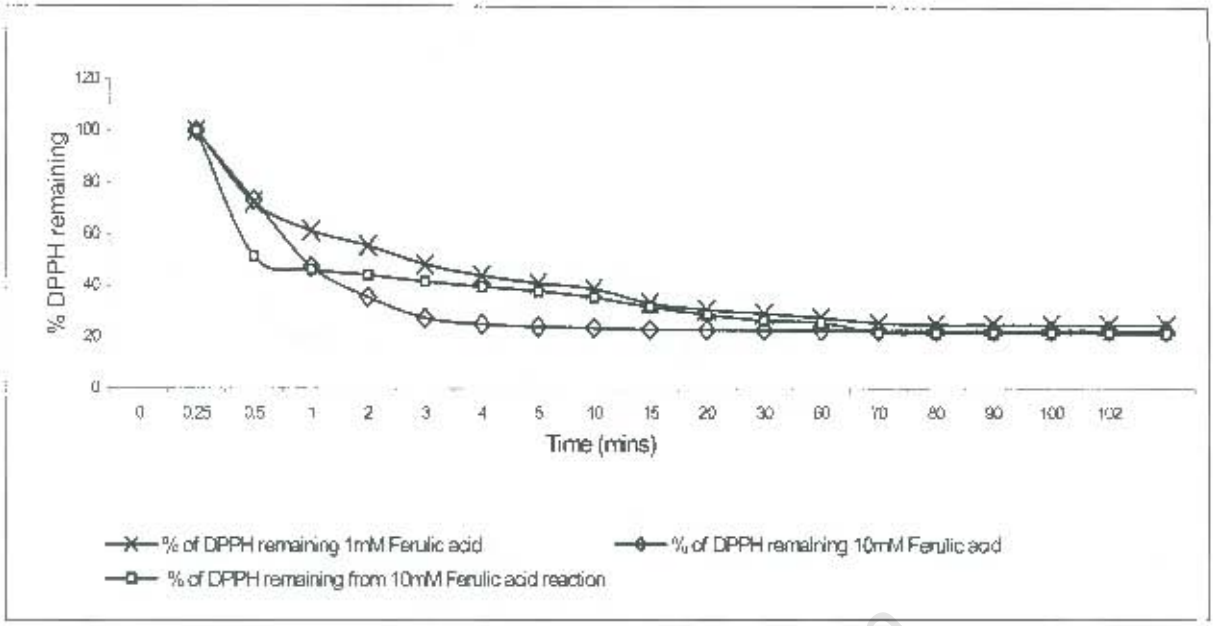


Figure 4-6. Assay of antioxidant capacity of ferulic acid-laccase reaction products, as compared with ferulic acid, using the DPPH assay.

4.3.3 TEAC antioxidant assay

The TEAC assay was conducted as described by Re *et al.* (1999). The extent of decolourisation of the $ABTS^{\cdot+}$ radical due to the scavenging of the radical cation by antioxidants was measured at 734 nm and the results plotted as shown in Figure 4-7. The addition of compounds synthesised during the course of this study resulted in a decrease in the absorbance, due to the quenching of the free radical by the antioxidants. The percentage quenching of $ABTS^{\cdot+}$ was determined by measuring the decrease in absorbance of the cation, and calculated as the percentage inhibition of absorbance at 734 nm (Re *et al.*, 1999). The reduction of the cation (which results in a decrease in absorbance) is dependent on antioxidant concentration and time (Re *et al.*, 1999; Antolovich *et al.*, 2002). Figure 4-8 and Table 4-4 show the percentage inhibition of absorbance elicited by the test compounds.

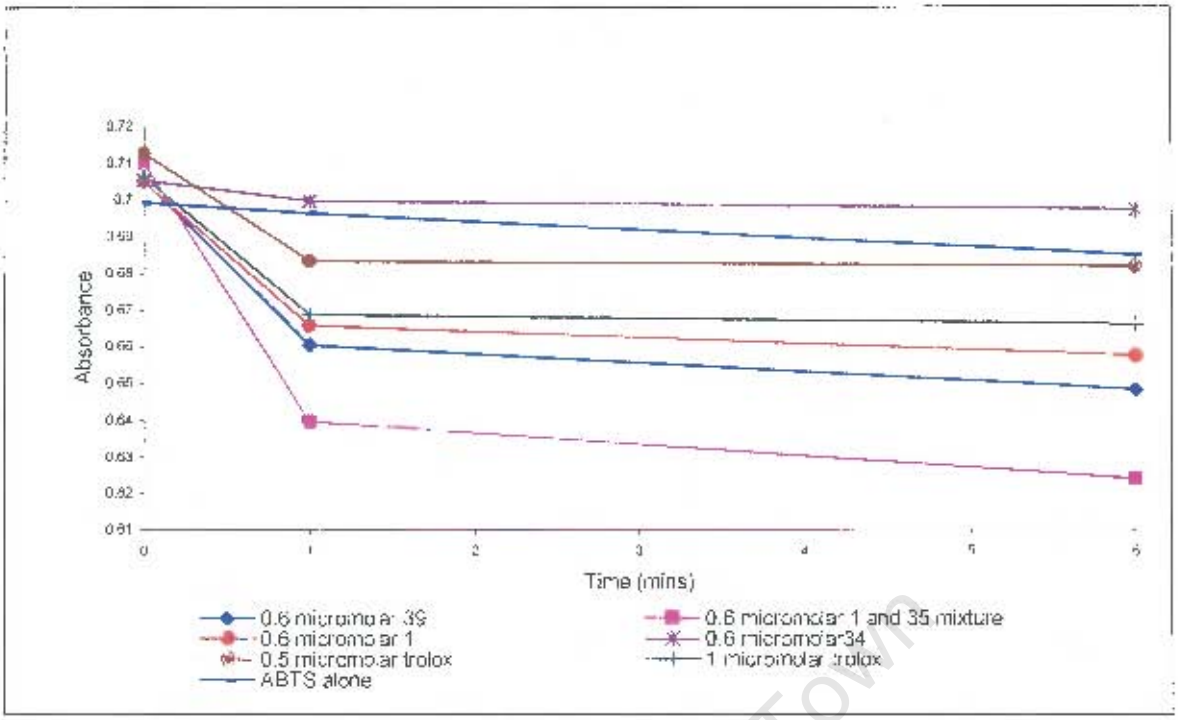


Figure 4-7. TEAC antioxidant assay: effect of the addition of test antioxidants on the absorbance of the ABTS $^{\bullet+}$ radical cation at 734 nm over time. A decrease in absorbance of ABTS $^{\bullet+}$ radical was due to scavenging of the radical by antioxidants.

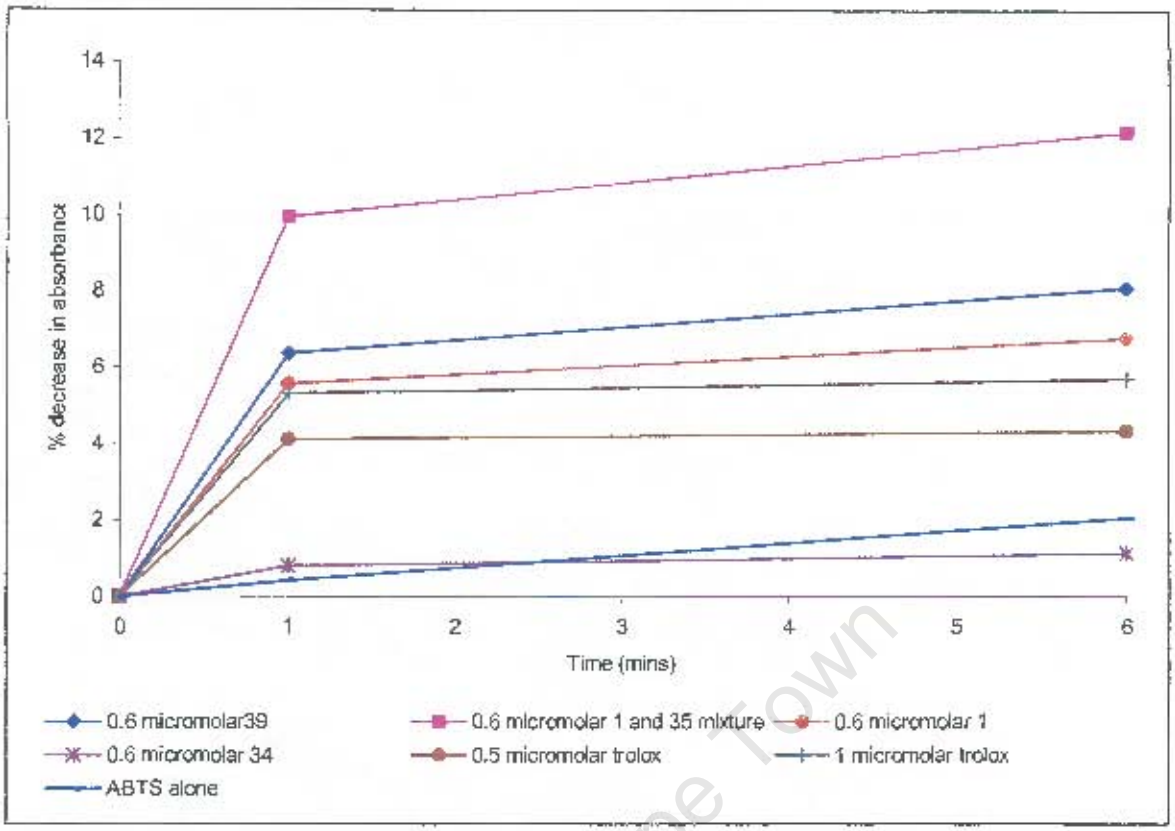


Figure 4-8. Changes in absorbance of ABTS$^{\bullet+}$ at 714 nm elicited by addition of 0.6 μM antioxidants and trolox over 6 minutes.

Table 4-4. Order of ABTS$^{\bullet+}$ radical cation scavenging ability of various compounds tested.

Rank	Compound	Compound final concentration	% inhibition of absorbance
1	1:4 **1,35** mixture	0.6 μM 1:4	12
2	**39**	0.6 μM	8.1
3	**1**	0.6 μM	6.8
4	Trolox	**1** μM	5.7
5	**35**	0.6 μM	4.94*[5]
6	Trolox	0.6 μM	<3.42*
7	**34**	0.6 μM	1.1

* calculated

The highest decrease in absorbance due to the ABTS radical cation was due to the addition of the 1:4 **1,35** mixture. The decrease was even greater than that of trolox at a higher concentration (Figure 4-8). The second highest decrease was elicited by the addition of product **39**, which was higher than that of ferulic acid, its precursor. The steroid, 5α,-androstane 3β,17β diol (**34**), by itself had very little inhibitory effect on the absorbance, (a maximum of 1.1 %). A calculated decrease of less than 10 % would be due to product **35** by itself[4]. If one assumed a synergistic effect was in effect as in the DPPH assay, the calculated decrease in absorbance would be 4.94 %[5], a value similar to that of ferulic acid (6.8 %), and trolox (5.7 %). These results are presented in Table 4-4.

To calculate the TEAC, the gradient of the graph of the percentage inhibition of absorbance vs. concentration plot ("concentration response curve") for the antioxidant in question was divided by the gradient of the plot for trolox (Re *et al.*, 1999). The concentration response curves for the antioxidant products were plotted as shown in Figure 5-8, and the TEAC values were calculated. This was done for compounds **1**, **35** and trolox. The TEAC of the mixture of **1** and **35** was higher than that of ferulic acid (Table 4-5).

Correlation was found between the DPPH and TEAC antioxidant activities in terms of free radical quenching ability, except for the arbutin ferulate product (**39**). This was expected because both methods measure the free radical quenching capacity of the test compounds using the same mechanisms. Thus, arbutin ferulate was found to have higher $ABTS^{\cdot+}$ free radical scavenging ability in comparison with the values obtained from the DPPH assay. Using the DPPH assay, the arbutin ferulate was also found to have a lower free radical quenching ability than ferulic acid, but its $ABTS^{\cdot+}$ scavenging was higher than that of ferulic acid. This indicates that product **39** has a better anti-radical activity

[4] This was calculated as the composition of the mixture was determined to be a 1:4 **1:35** ratio from NMR analysis. Therefore the decrease due to **35** was < 0.8x that of the mixture. It was less than 0.8x of the mixture as antioxidant synergy between **1** and **35** was demonstrated in the DPPH assay. As a result the activity due to **35** by itself would be less than the calculated value, as more than an additive effect accounts for the synergistic activity.

[5] The TEAC activity of **35** alone considering identical synergistic effect to that observed with DPPH assay=Synergistic factor x calculated non synergistic TEAC value of **35**.
Synergistic factor= Calculated DPPH activity / actual DPPH activity observed

against the ABTS free radical than the DPPH free radical. The differences between the activities of arbutin ferulate in the different assays can be explained by the "polar paradox" (Porter, 1993). The polar paradox explains that "polar antioxidants are more active in lipophilic mediums whereas lipophilic antioxidants are more active in polar mediums" (Brand-Williams *et al.*, 1995). Arbutin ferulate was found to be a very polar compound from TLC analysis during its synthesis and the steroid compounds were found to be lipophilic (Table 4-6). The polarity of arbutin ferulate is conferred by the sugar moiety in its structure, and the steroid derivatives are non-polar due to their steroidal parent compounds which are lipophilic. The TEAC assay is performed in buffered water, and the DPPH assay is conducted in methanol which is less polar (more lipophilic) than water (water has a polarity index of 9 and methanol has an index of 6.6 according to the Snyder polarity index, http://home.planet.nl/~skok/techniques/hplc/eluotropic_series_extended.html#3). As a result, arbutin ferulate would be expected to be more active in the TEAC assay (performed in a polar medium) than in the DPPH assay (performed in a more lipophilic medium). The same effect was observed by Brand-Williams *et al.*, (1995) when they compared antioxidant activities obtained using the DPPH assay and the methyl linoleate assay, an assay conducted in more lipophilic conditions than the former.

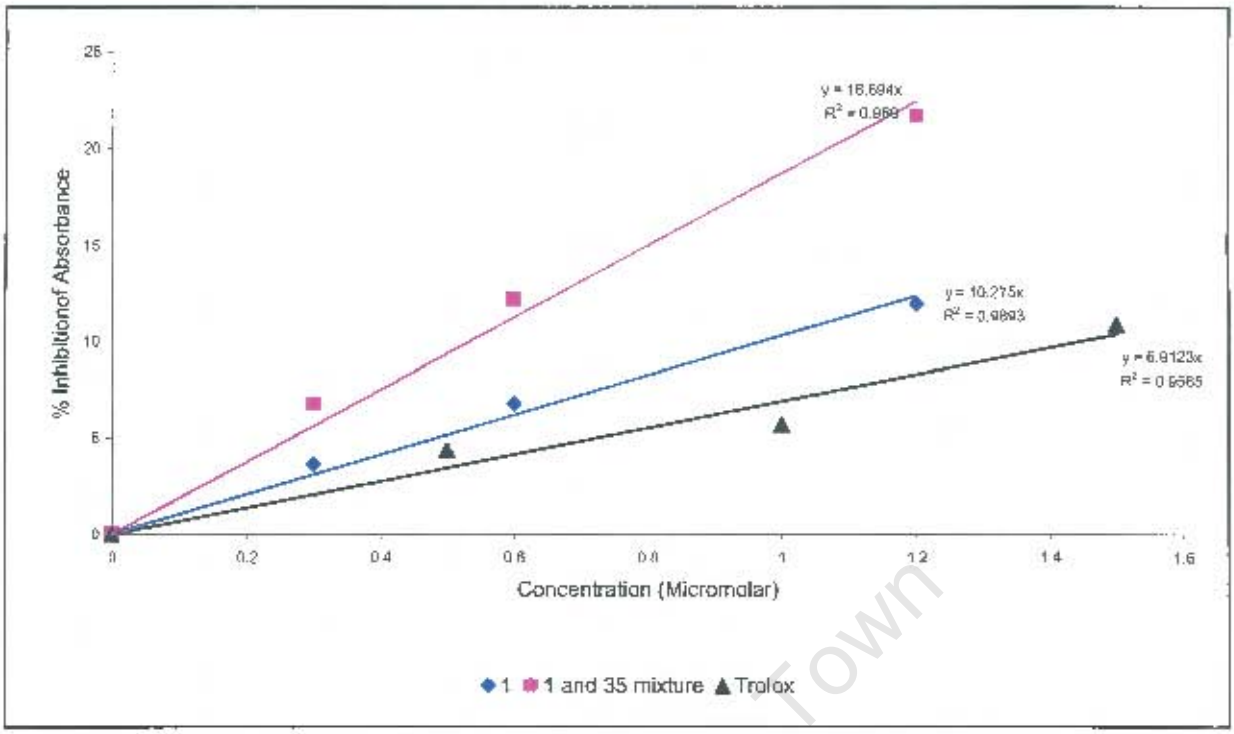


Figure 4-9. Effects of the antioxidant concentration on the inhibition of absorbance due to the reduction of $ABTS^{\cdot+}$ (concentration response curves).

Table 4-5 The measurement of the quenching of the $ABTS^{\cdot+}$ free radical by various antioxidants and the corresponding TEAC.

Compound	Maximum % decrease of Absorbance (0.6 μM) after 6 minutes	TEAC μM
Trolox	4.15	1
1	6.76	1.49
1:4 **1,35** mixture	12.15	2.70
35	10	2.16*

**calculated*

4.3.4 Inhibition of LDL oxidation initiated by Cu^{2+} by antioxidants

The ability of the biocatalytically synthesised compounds to inhibit LDL oxidation was assessed by adding them to a reaction mixture containing copper (to initiate oxidation)

and LDL, and then noting the effect on the lipid peroxidation-associated diene conjugation. An increase in the absorbance at 234 nm indicated diene conjugation. The results of the reactions are reported below in Figure 4-10. Of the compounds tested, arbutin ferulate (**39**), was found to confer the highest inhibition of LDL oxidation, higher than that of its precursors, ferulic acid (**1**), and arbutin, (**38**). Therefore, the esterification of arbutin with ferulic acid increased the arbutin antioxidative activity towards copper induced oxidation of LDL by 10 %. The compound **35**, 3β–O-Feruloyl-17β-hydroxy-5α-androstane also had an antioxidative effect as did the steroid from which it was derived from, compound **34**.

All of the antioxidants tested had an absorbance-decreasing effect, (prevention of diene conjugation due to lipid peroxidation) except for the 1:4 ferulic acid; 3β–O-feruloyl-17β-hydroxy-5α-androstane mixture, and dihydrocholesterol ferulate (**33**). It was unexpected that the mixture would have an absorbance-increasing effect indicating accelerated diene congugation and further investigations into these results would need to be pursued in order to provide an explanation. It was also interesting to observe the increased oxidation effect (indicative of formation of diene conjugates due to lipid peroxidation) of dihydrocholesterol ferulate **33**. This may be explained on the basis of the structure and chemistry of LDL particles. LDL particles are composed of cholesterol esters, which are hydrophobic and are encapsulated by a layer of phospholipids and unesterified cholesterol, which is much more polar than the esters (http://www.lipidlibrary.co.uk/Lipids/sterols/index.htm#ce, illustrated in Figure 4-11 below). The polar theory could be used to explain the low activity observed for compound **33**. It is a very lipophilic compound and the LDL particles in which it was to exert antioxidant activity are also lipophilic. As a result it exerted no protective effect on LDL oxidation. Hence, the increase in absorbance was due to the oxidation of LDL which took place, uninterrupted by compound **33**, as it had no activity when exposed to lipophilic conditions. This hypothesis is supported by the TLC results obtained in the course of this study, which showed that compound **33**, was a highly non-polar compound (Table 4-6 tabulates the R_f values obtained from TLC of the products using 10:1 chloroform:ethyl acetate obtained during the course of this study) with an R_f value of 0.7

compared to that of **35** (R_f of 0.25). It is proposed that this product, due to its non-polarity, enters into the lipophilic LDL particle lipophilic core with the other ferulic acid esters, preventing it from exerting much antioxidant activity as explained by the polar theory. The LDL oxidation mechanism is not understood and further knowledge would provide insight into the results obtained for the product **33** and the ferulic acid product **35** mixture (Noguchi 2002; Graßmann *et al.*, 2005). Other triterpene androgens and progestins such as testosterone and progesterone were found to have pro-oxidant activity on LDL as they accelerated the rate of LDL oxidation initiated by Cu^{2+}(Zhu *et al.*, 1999) as observed for compound **33**. Therefore, lipophilic compounds, such as compound **33**, are poor inhibitors of LDL oxidation, a finding which has significant repercussions on therapeutic *in vivo* LDL oxidation inhibitors.

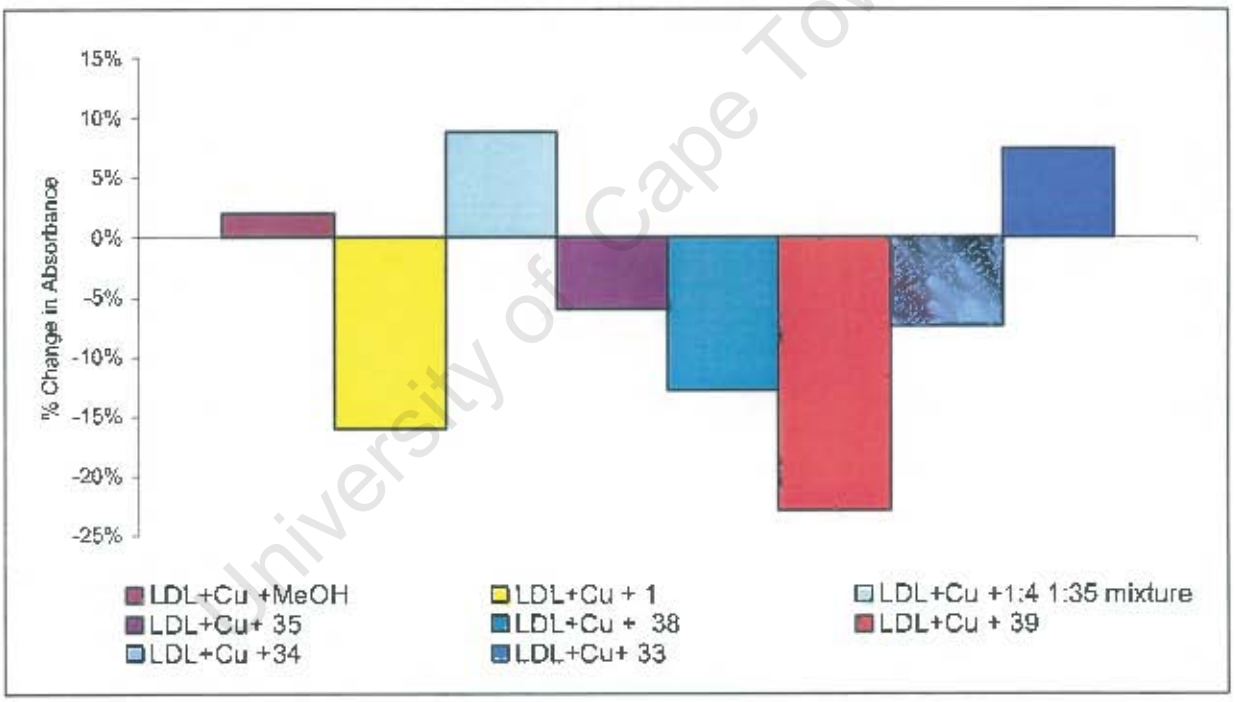


Figure 4-10. Changes in absorbance due to the Cu^{2+} induced formation of conjugated dienes in LDL samples. An increase in absorbance indicated the formation of conjugated dienes and a negative change indicted an inhibition of the formation of conjugated dienes.

Table 4-6. R_f values of the steriod derivative compounds in descending order of polarity obtained from TLC using 10:1 chloroform:ethyl acetate as mobile phase.

Compound	Compound name	R_f value
1	Ferulic acid	0.075
34	5α Androstane 3β, 17β diol	0.05
35	3β–O-Feruloyl-17β-hydroxy-5α-androstane	0.25
27	Dihydrocholesterol	0.325
33	Dihdrocholesterol ferulate	0.7

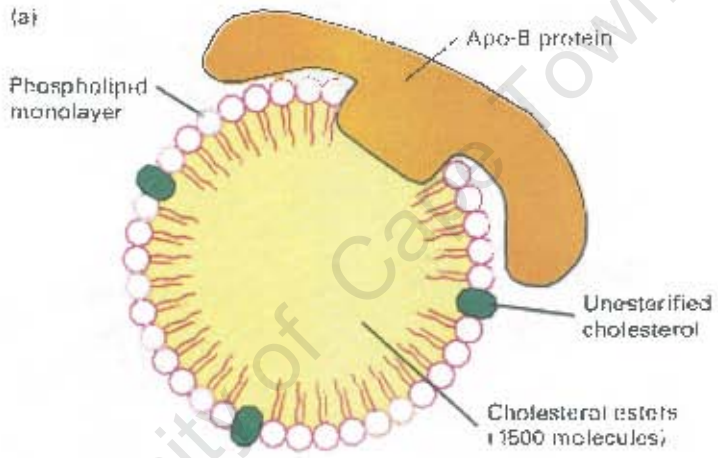


Figure 4-11. Schematic diagram of an LDL particle. An outer monolayer of phospholipids and unesterified cholesterol covers a hydrophobic cholesterol fatty acid ester and triglyceride core. Embedded in the phospholipid and cholesterol layer, is the protein Apo-B. (Figure taken from Lodish *et al.*, 2000).

4.3.5 NADH oxidase assay.

The cofactors NADH and NADPH have been reported to be intramolecular antioxidants in the cell cytosol (Kirsh and Groot, 2001), and these compounds are hydride donors in many enzymatic processes. They are recognised as indirectly-acting antioxidants in this role, as they are involved in the reduction of proteins such as glutathione, some of which have antioxidant functions. Evidence has emerged that NADH and NADPH themselves can act as directly operating antioxidants; they have been reported to quench radicals derived from peroxynitrite and other unstable free radicals and to "repair" harmful radicals such as glutathiyl and tyrosyl radicals (Kirsch and Groot, 2001). This independent (directly operating antioxidant) function complements the coenzyme (indirectly operating antioxidant) role.

The enzyme NADH oxidase (NOX), converts NADH to NAD^+. It is a membrane associated protein, reported to be responsive to anticancer drugs. Studies have suggested that several phenols in wine could prevent the oxidation of NADH from peroxynitrite, a free radical that is linked to various human diseases (Valdez *et al.*, 1998). A tumour-associated NOX gene, tNOX, has been identified which was found to be upregulated in and associated with cancers. Therefore, it was suggested that phenols which can inhibit NADH oxidase could play a major role in the inhibition of the expression of NOX genes, thereby decreasing uncontrolled growth of tumour cells in cancer (Morre *et. al.*, 1995). Based on these and other findings, a hypothesis was proposed in this study, that the inhibition of NADH oxidase is a physiologically relevant antioxidant assay. As a result, several compounds, including those produced biocatalytically during this study, were assessed for their ability to inhibit NADH oxidase (NOX) activity. A bacterial, water-forming NOX isolated from *Lactobacillus sanfrancisnensis* was used for the assays described in this study. *L. sanfrancisnensis* NOX accepts both NADPH and NADH as substrate (Lountos *et al.*, 2005). NADH was used as a substrate and the enzyme activity assay was conducted according to Riebel *et al.*, 2002. NOX was added to NADH in TEA buffer and the decrease in NADH levels was measured spectroscopically at 340 nm.

Decrease in NOX activity over time was noted and hence all results show NOX activity at specific times.

4.3.5.1 Effect of solvent on the NOX assay

The phenolic compounds used in this study did not dissolve well in water, but they dissolved well in organic solvent. Thus, an investigation into the effect of two organic solvents was conducted in order to find one that did not interfere with the NOX assay. Ethyl acetate was found to inhibit enzyme activity at 1 % v/v whereas methanol did not at 1 % or 3 % v/v (Figure 4-12). Subsequently, all compounds were dissolved in methanol.

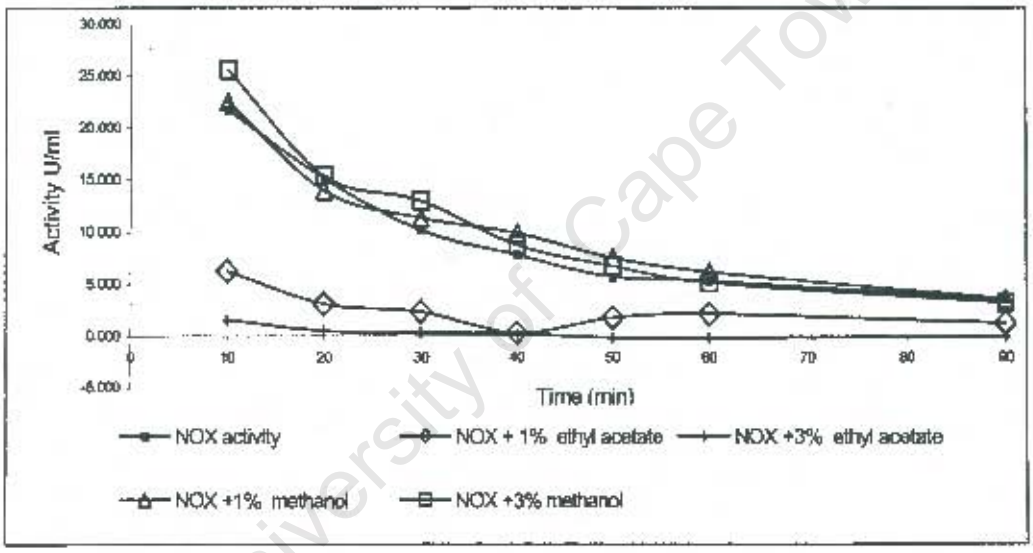


Figure 4-12. Effect of ethyl acetate and methanol on NOX activity. Methanol was found to have no interference at 1 and 3 % v/v on the assay and was used to dissolve all compounds used.

4.3.5.2 Phenolic compounds with no inhibitory effect on NOX activity

Addition of some phenolic compounds to NADH solution did not increase the absorbance of the solution at 340 nm. These compounds were also found not to affect NOX activity, when they were added to the assay mixture. These compounds were *p*-arbutin, catechin, catechol, eugenol and ascorbic acid. The decreases in absorbance due to NOX activity,

were the same for NOX with or without the addition of these compounds (Figure. 4-13). Thus, these compounds did not affect NOX activity significantly (Figure. 4-14).

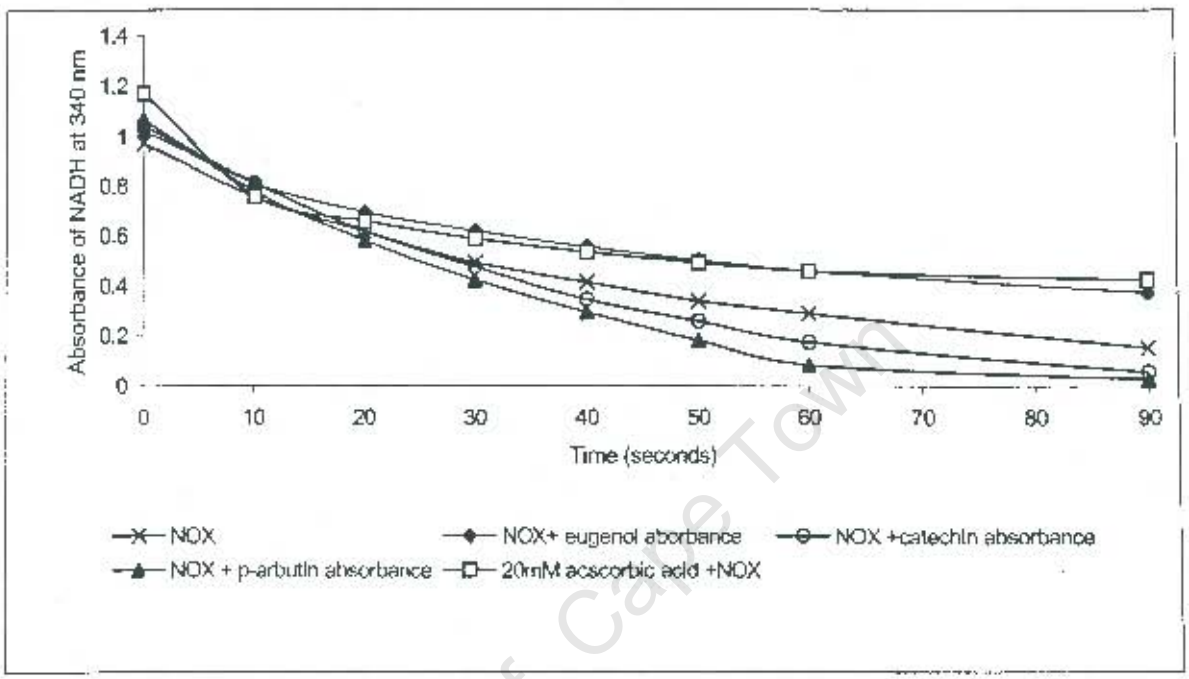


Figure 4-13. Effect of the addition of non-NOX inhibiting phenolic compounds in NOX assay on absorbance. The absorbance at 340 nm did not change when 10 mM catechin (a flavonoid), eugenol, *p*-arbutin or 20 mM ascorbic acid was added.

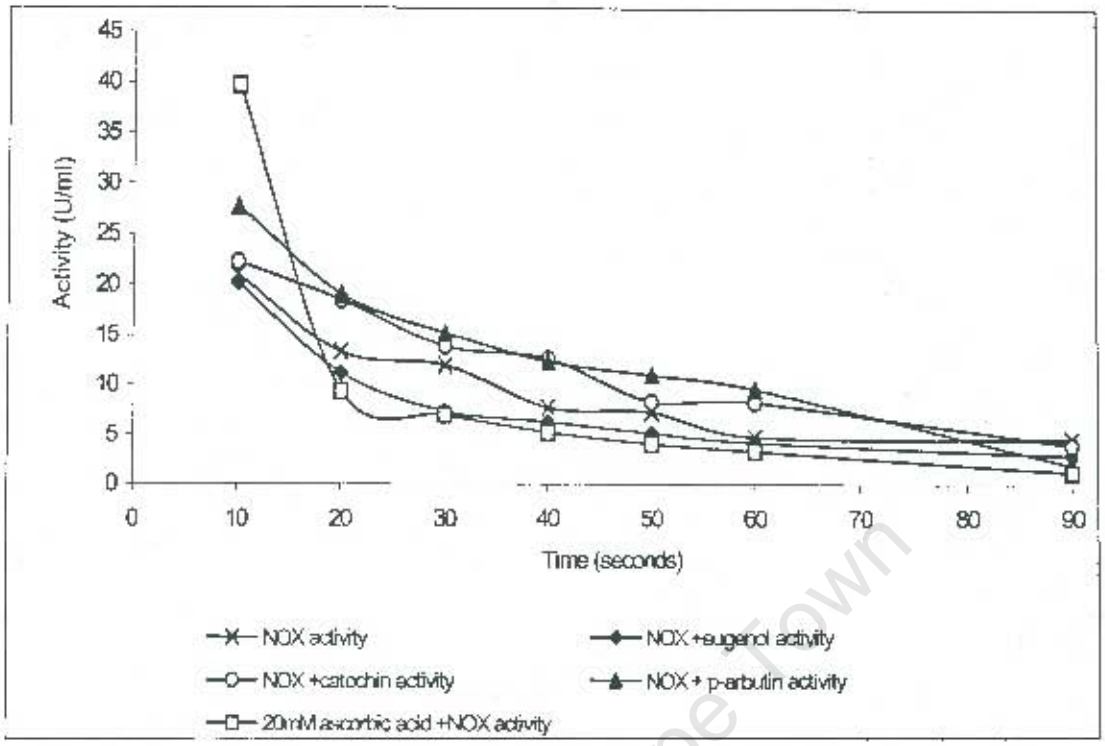


Figure 4-14. Activities of NOX after addition of non-inhibiting phenolic compounds. NOX activity was not significantly affected by the addition of 10 mM catechol, catechin, eugenol, p-arbutin or 20 mM ascorbic acid

4.3.5.3 Effect of phenolic compounds with putative inhibitory effect on NOX activity

The assay used in this study detected NADH levels via its absorbance at 340 nm, since NADH absorbs at that wavelength but oxidised NAD^+ does not. Several of the compounds that were assayed, upon addition to NADH, resulted in an increase in the absorbance of the reaction mixture. These were termed "Absorbance increasing compounds". Figure 4-15 shows the effect of these compounds on the absorbance. Most of these increased the absorbance by an amount much more than their own absorbance at 340 nm.

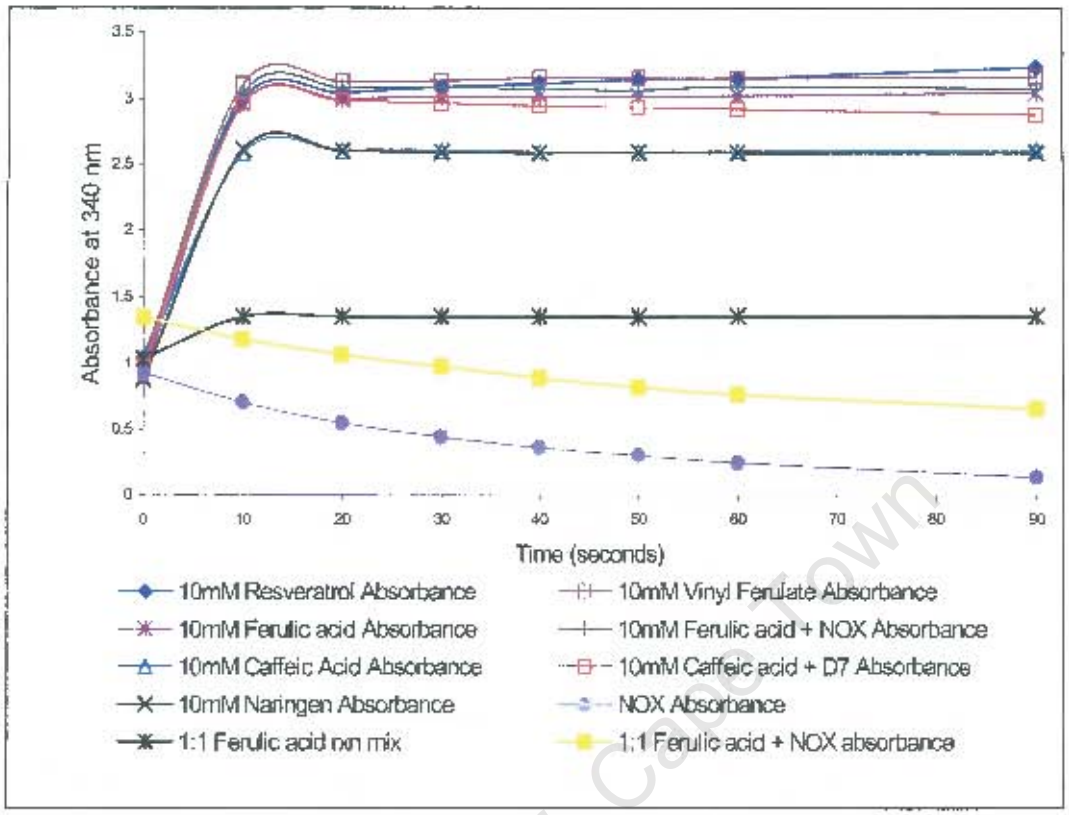


Figure 4-15. Effect of putative NOX inhibitors on NADH absorbance at 340 nm. 10 mM ferulic acid, naringen, caffeic acid, vinyl ferulate, resveratrol and products from a 10 mM ferulic acid reaction caused an increase in absorbance at 340 nm. D7 was the name of the NOX used.

It was considered that an increase in absorbance at 340 nm may have indicated increased levels of NADH. In order to ascertain this, NOX, which converts NADH to NAD^+, was added to the mixture. However, the increase in absorbance due to the addition of 10 mM phenolic compounds was not significantly affected by the addition of the NOX enzyme to the reaction mixture. This may have been due to an increase in NADH concentration to levels that were inhibitory to the enzyme, or more likely due to absorbance-increasing complexes of NADH with phenolic acid compounds having been formed, that were not substrates of the enzyme used. An addition of NOX did not alter the increase in absorbance observed.

As an alternative explanation, the increase in absorbance at 340 nm caused by the addition of acidic phenolic acids (caffeic and ferulic acids) could have been due to either a chemical reaction between residual NAD^+ in the NADH, or a reaction between the NADH and the phenolic acids. To test this hypothesis, NAD^- was reacted with the respective absorbance-increasing compounds. This did result in an increase in absorbance at 340 nm (NAD^+ does not have an absorbance at that wavelength). This may possibly have been due the formation of NAD-phenolic complexes which absorb at 340 nm. Acidic phenolic acids such as ferulic acid and caffeic acid showed this absorbance increasing effect, whereas eugenol (which is identical to ferulic acid except for the carboxylic acid moiety on the *para*-substituted subunit) did not. Thus, eugenol does not have the carboxylic group that would react with NAD^- and so does not cause an increase in absorbance whereas caffeic acid does. Caffeic acid has the *para*-substituted carboxylic acid group like ferulic acid and has an identical effect.

The chemical reaction that explains this is illustrated in Figure 4-16. The investigation into the effect of the methyl group or the methoxy group on the phenolic ring was conducted using catechol. Catechol has the same phenol ring with a methoxy group on it, but unlike caffeic acid it does not have the *para*-substituted moiety. Catechol did not have an effect on the NADH, again suggesting that the acidic moiety is responsible for the increase in absorbance effect.

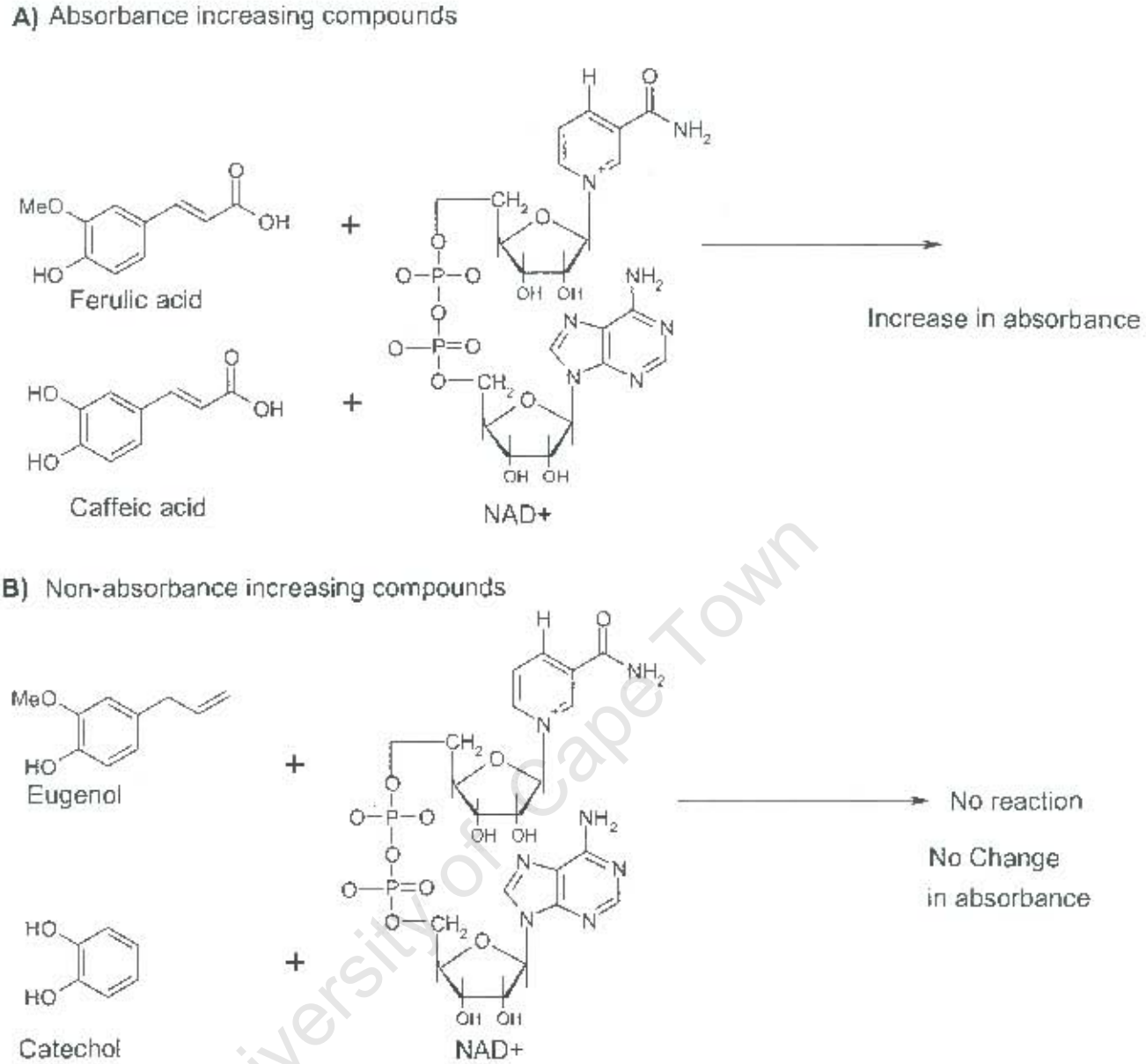


Figure 4-16. Effect of selected compounds on NAD^+. A) Addition of phenolic acids such as ferulic acid to NAD^+: An increase in absorbance was observed. It is postulated that the acids react with NAD^+ leading to the formation of a phenolic-NAD complex which absorbs at 340 nm like NADH. B) Addition of non-acidic phenolic compounds where no change in absorbance at 340 nm was observed.

4.3.5.4 Effect of the addition of the organic phase reaction products of 10 mM ferulic acid reaction with immobilised laccase in biphasic reaction system: addition of NOX after test compounds

The effect of the laccase synthesized products of ferulic acid on NOX was investigated. The reaction products in the organic phase of the biphasic (ethyl acetate/sodium acetate buffer) 10 mM ferulic acid reaction mixture with laccase that had been immobilised at pH 7 were used. The synthesis is explained in Chapter 2, Section 2.3.7.2 and product characterisation in section 2.4.7.1. The products were characterised to be 4-vinylguaiacol (**10**) (section.2.4.7.1) and small quantities of ferulic acid dimers. The effect of this product mixture on NOX activity was investigated. The reaction products from the conversion of 0.2 mM and 0.02 mM[6] ferulic acid by laccase were tested. These concentrations were equal and 0.1x the concentration of NADH used.

The product mixtures were added to NADH solution as previous in order to determine whether an increase in absorbance would result. There was an increase in absorbance due to the product mixtures, as in the case of ferulic acid which is their substrate (Figure 4-16)[7]. Unexpectedly, the difference between the increases in absorbance of the respective concentrations was minimal. The overall increase of both concentrations was small and was easily reversed by the addition of NOX enzyme. Thus, the results showed that there was no inhibitory effect on NOX activity due to the ferulic acid–laccase reaction products, at the concentrations tested (Figure 4-17).

[6] Conversion of 10mM ferulic acid was conducted and a volume equivalent to 0.2 and 0.02mM ferulic acid converted was used.

[7] "Activities" of compounds were calculated using the extinction co-efficient of NADH used for the enzyme assays. This was done in order to have a comparison for levels of increase noted for each compound and to observe the effect the addition of NOX would have on the high absorbance's obtained.

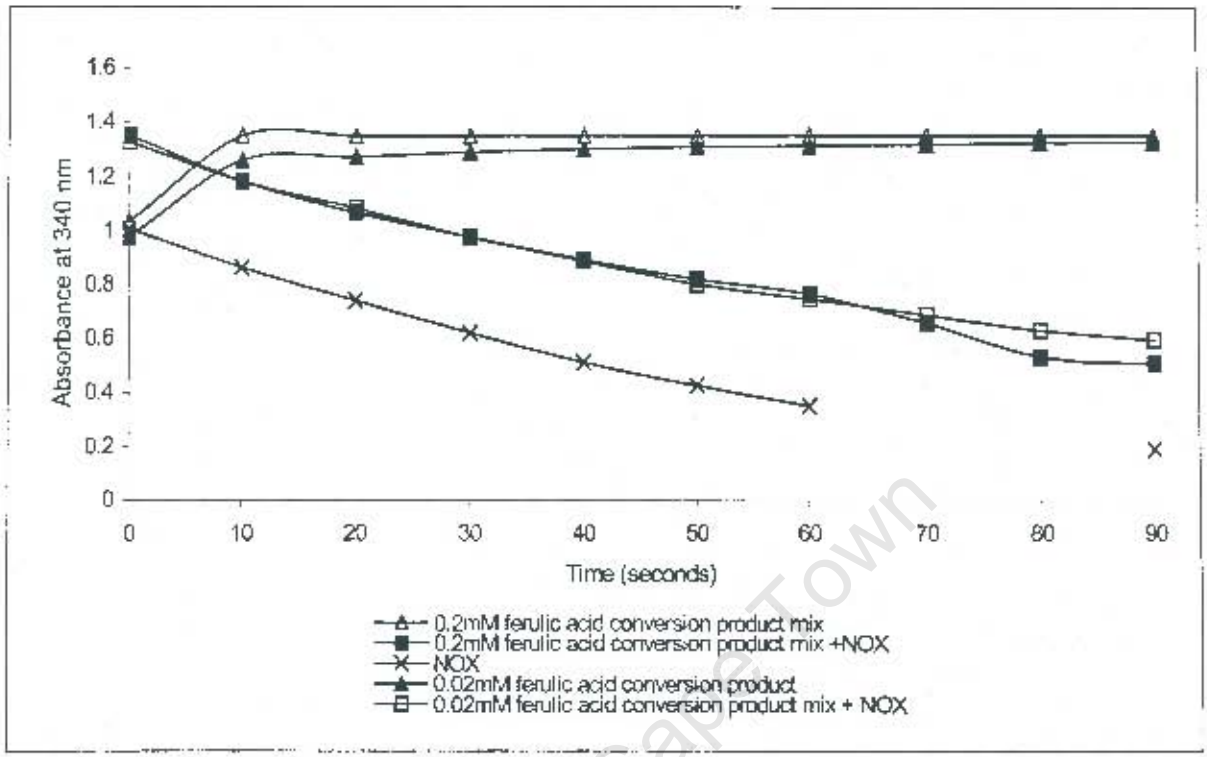


Figure 4-17. Effect of laccase/10 mM ferulic acid reaction on absorbance of NADH solution at 340 nm. There was no significant difference between the increases in absorbance caused by an equal or 0.1x concentration of ferulic acid reaction conversion products.

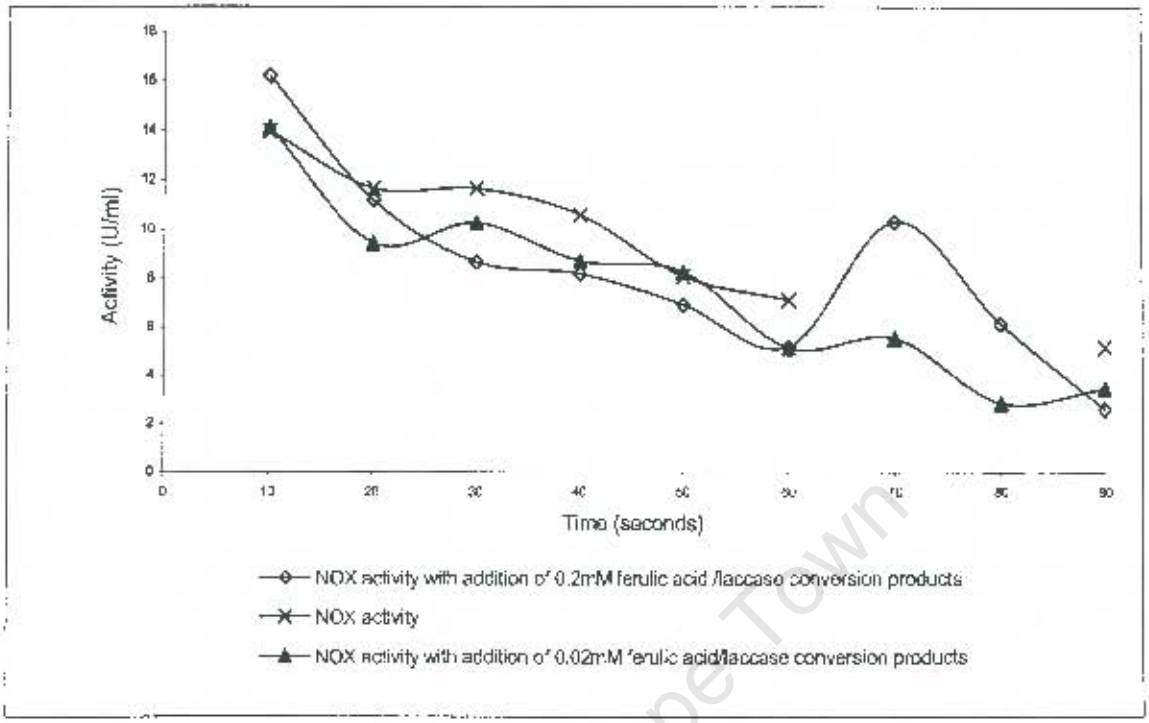


Figure 4-18. Effect of laccase/10 mM ferulic acid reaction product mixture on NOX activity. NOX activity was not affected by the addition of the products, as the NOX activity curve was unaffected as shown.

4.3.5.5 Investigation into the effect of different substrate mixing sequences on NOX activity: adding resveratrol simultaneously, or before the addition of NOX

There was a significant difference observed between the NOX activities obtained when resveratrol was added simultaneously or before the addition of NOX. Two concentrations of resveratrol were used during the investigation, equal to or 0.1x the concentration of NADH used in the assay. Figure 4-18 below shows the results obtained. The addition of resveratrol resulted in an increase in absorbance, which was inversely proportional to activity. This resulted in negative "activities" being calculated for resveratrol, which were concentration dependant as shown. Addition of NOX after mixing 0.2 mM and 0.02 mM resveratrol with NADH resulted in a decrease of 42.8 and 26.3 % NOX activity respectively. The simultaneous addition of NOX and 0.2 mM resveratrol resulted in an

overall negative activity. The increase in absorbance from the resveratrol only was greater than when NOX was added simultaneously, indicating NOX activity taking place in the presence of 0.2 mM resveratrol. When 0.02 mM resveratrol was added simultaneously with NOX there was an overall positive NOX activity which was 37.3 % lower than NOX activity alone.

Table 4-7. The effect of adding 0.02 and 0.2 mM resveratrol on NOX activity

	Enzyme alone	Resveratrol	
		0.2 mM	0.02 mM
Compound alone	16.6 U/mL	-30 U/mL	-9.1 U/mL
Simultaneous addition of compound with enzyme		-25.2 U/mL	10.4 U/mL
Difference in NOX activity		-251%	-37.3 %
Addition of compound first then enzyme after 40s		9.5 U/mL	12.4 U/mL
Difference in NOX activity		-42.8 %	-26.3 %

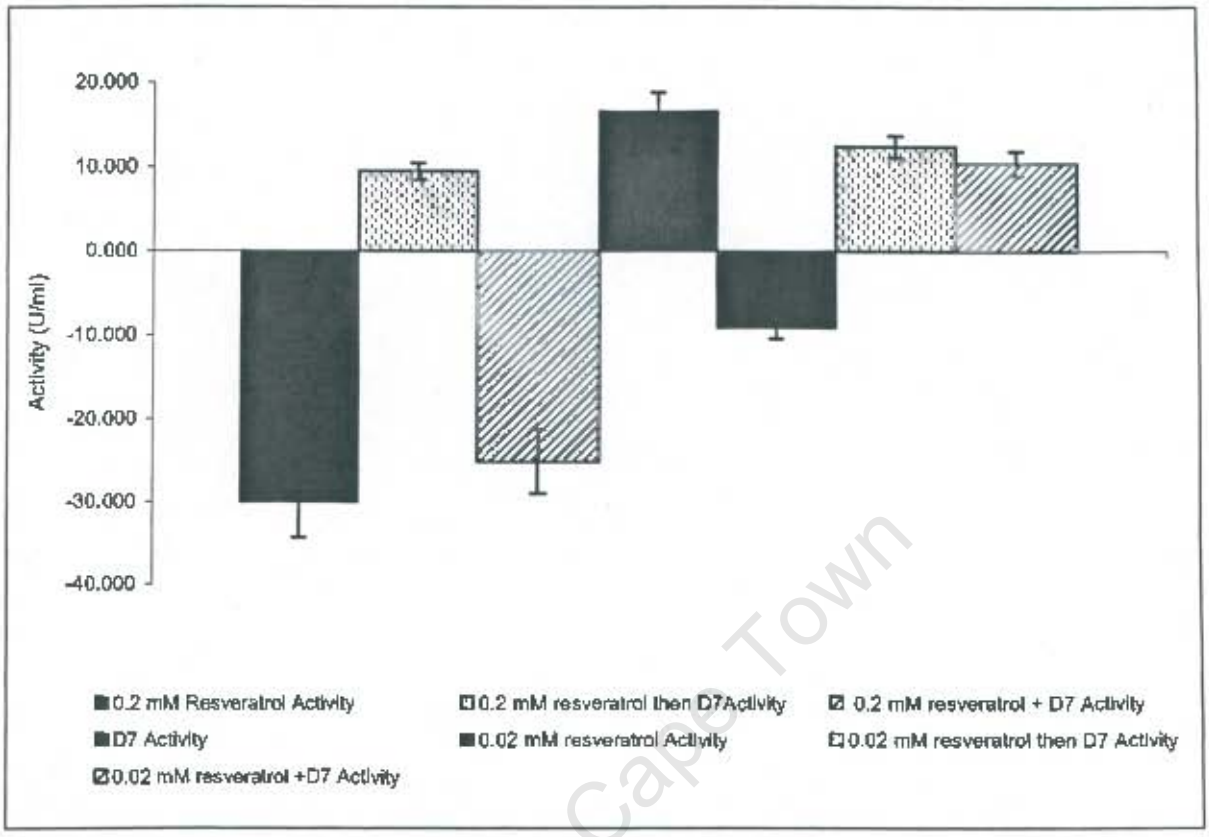
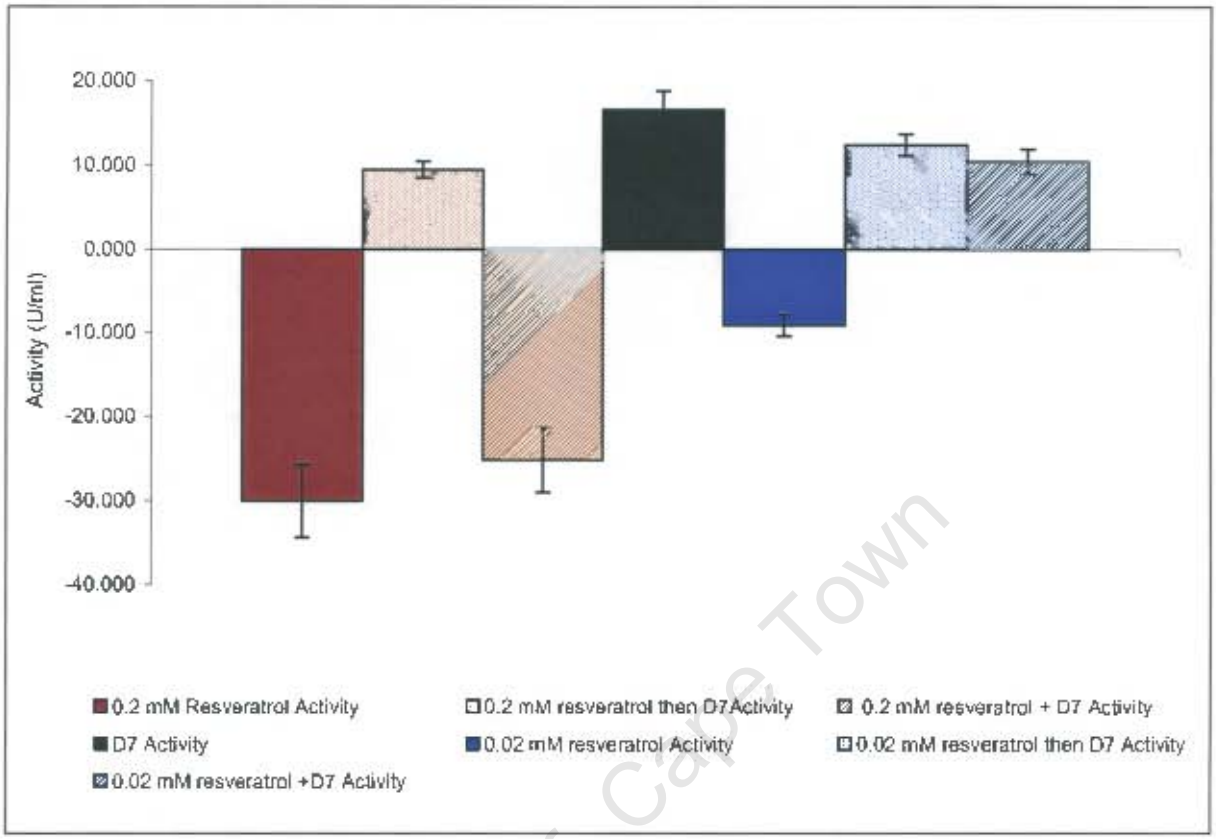


Figure 4-19. Inhibition effects of phenolic acids on NOX activity using 0.2 mM NADH and 0.2 mM or 0.02 mM phenolic compound inhibitors.

Using a 1:10 ratio of 0.02 mM resveratrol and NADH, there was less difference between the NOX activities when resveratrol was added simultaneously or added subsequent to the addition of NADH (37.3 % and 26.3 % respectively). This result corresponds well with a previous report of the inhibition of 25-30 % of pig liver transitional endoplasmic reticulum NOX, by retinol (Sun *et al.*, 2000).

Orallo *et al.*, (2002) reported a 71 % decrease of endothelial containing Rat aortic NOX activity by 10 μM resveratrol. The low inhibition values obtained in this study are attributed to several factors, most significantly concentration effects. The reported studies used mammalian NOX proteins in tissue homogenates, which would have contained low NOX concentrations. In this study, recombinant bacterial protein used was pure and protein concentrations used were those used in previous standard NOX assays in which



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the enzyme was obtained (Riebel *et al.*, 2002). Those conditions may have utilised NOX concentrations that were too high in proportion to the substrate NADH and inhibitor concentrations.

4.3.5.6 Investigation into interruption of NOX activity by ferulic acid organic and aqueous conversion products

The results of the previous section showed a difference in the extent of NOX inhibition dependent on the order of reagent addition. This experiment investigated the effect of adding the inhibitor after uninhibited NOX activity had been initiated. NOX activity was allowed to proceed for 40 seconds before the putative inhibitors were added. The 10 mM ferulic acid conversion products synthesised in a biphasic reaction catalysed by immobilised laccase were used to carry out the investigation. Both the organic and aqueous fractions were tested. In Section 5.3.4.4 it was reported that the laccase/ ferulic acid products did not have a marked effect on NOX activity at the concentrations that they were tested when NOX was added after the products (Figure 4-16 and 17). However, a marked inhibition of NOX activity was observed after addition of the ferulic acid reaction mixture. An initial dip in NOX activity was observed immediately after the addition of the reaction mixture (at 40s), and the enzyme activity did increase again, but not to original NOX activity levels (Figure 4-19).

Table 5-8 shows the NOX activities before and after the addition of the reaction mixture. Inhibition was defined and calculated as the increase in the ratio of residual NOX activity after inhibitor addition, over the original initial activity, compared to that of uninterrupted NOX, in order to normalise the data. The ratios were calculated based on the initial ferulic acid concentration of the reaction. The final NADH concentration was 0.2 mM; therefore the 1:10 ratio used contained 0.02 mM initial ferulic acid and the 1:5 ratio, 1mM initial ferulic acid. Inhibition was observed for the aqueous fraction of the reaction used. This was due to the ferulic acid polymers being present in the aqueous phase of the reaction and not the ethyl acetate. The 1:10 ratio of the organic phase of the reaction

containing 4-vinylguaiacol (which is more soluble in ethyl acetate) was found to be less inhibiting of NOX activity.

It can be concluded that ferulic acid polymers possess higher NOX inhibitory effect (30.76 %) than the 4-vinylguiacol (9.39 %). Ferulic acid itself exhibits NOX activity inhibition, which can be increased by its polymerisation as reported by others and in this present study. The antioxidant activity of ferulic acid is increased by its polymerisation and it follows that its NOX inhibition would also be increased, since its mechanism of inhibition may be linked with its antioxidant activity. This hypothesis would need further investigation to assess its validity.

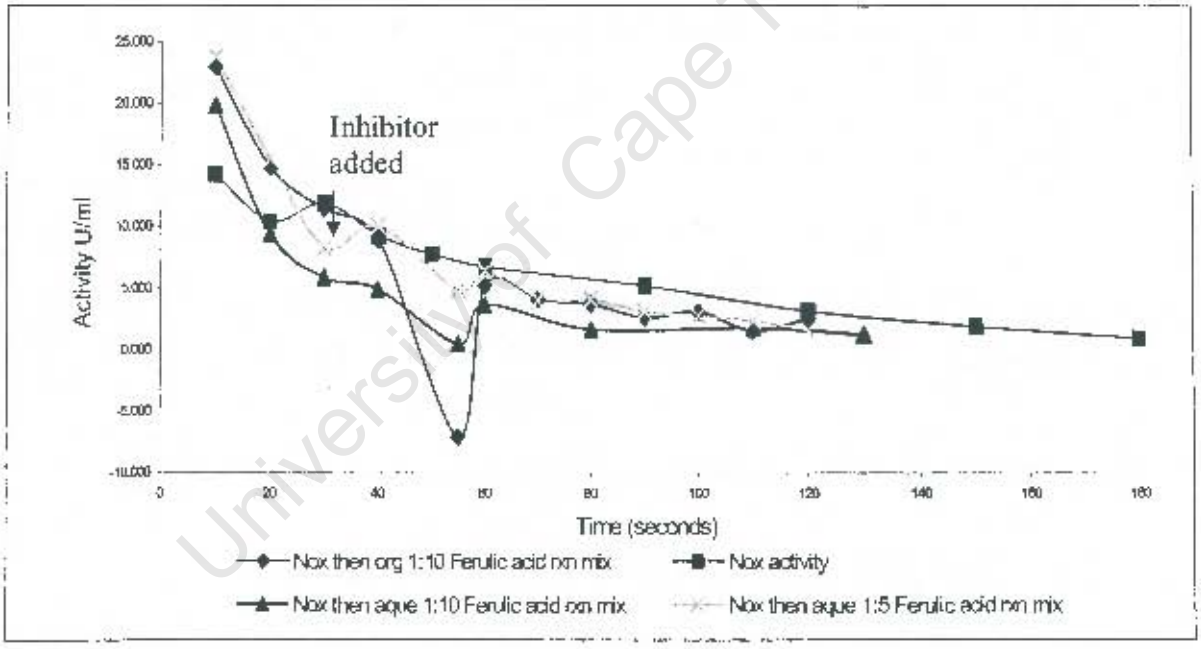

Figure 4-20. Effect of addition of 10 mM ferulic acid reaction with immobilised laccase in biphasic reaction system reaction mix after 40 seconds of NOX activity over time. The activity of the NOX activity was inhibited by the addition of the ferulic acid reaction mix. The decrease in activity between 50 and 60 seconds was due to the initial increase in absorbance due to the addition of the test compound, which is then quenched.

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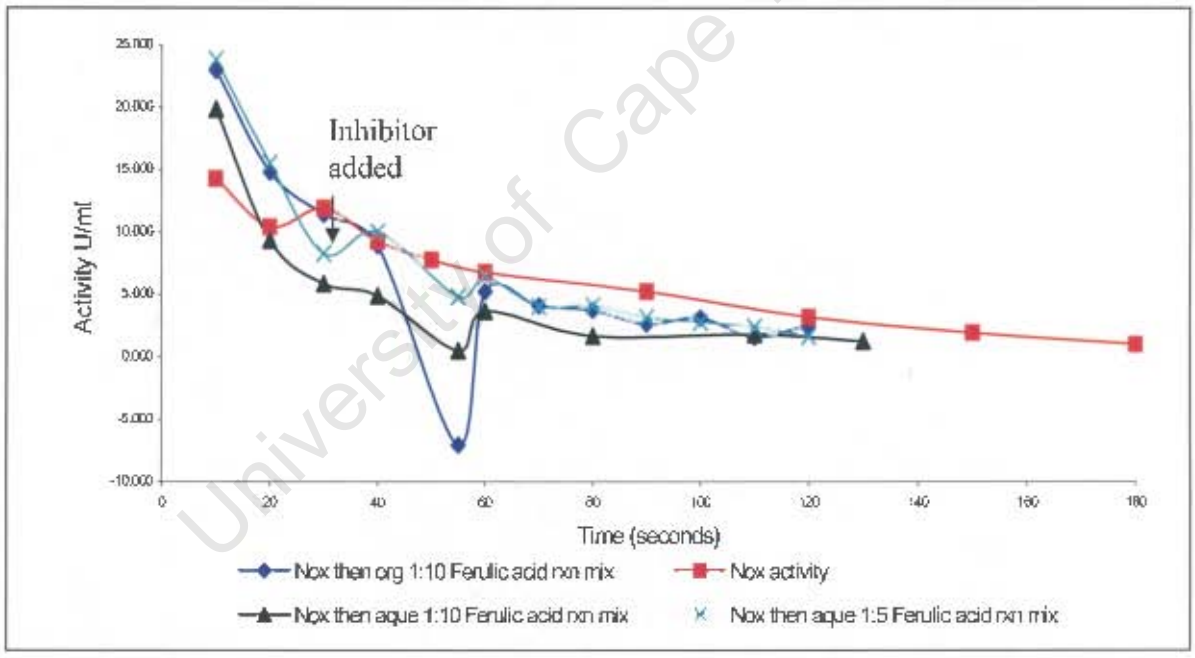
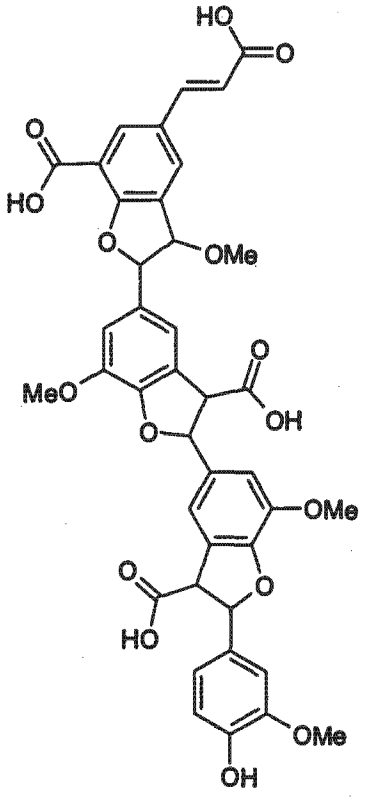


Figure 4-20. Effect of addition of 10 mM ferulic acid reaction with immobilised laccase in biphasic reaction system reaction mix after 40 seconds of NOX activity over time. The activity of the NOX activity was inhibited by the addition of the ferulic acid reaction mix. The decrease in activity between 50 and 60 seconds was due to the initial increase in absorbance due to the addition of the test compound, which is then quenched.

Table 4-8. Effect of the interruption of NOX activity by 4-vinylguaiacol and ferulic acid polymers

Sample	Initial NOX activity before addition of inhibitor A	Residual NOX activity after addition of inhibitor B	A/B	% Decrease in NOX activity
NOX under normal assay conditions	11.9 U/ml	5.1 U/ml	2.86	
NOX activity after addition of aqueous phase of 0.04 mM ferulic acid conversion products (composing different isomers of ferulic acid polymers) (1:5 ferulic acid:NADH ratio)	14.436 U/ml	3.6 U/ml	4.01	40.2 %
NOX activity after addition of aqueous phase of 0.02 mM ferulic acid conversion products (composing different isomers of ferulic acid polymers) (1:10 ferulic acid:NADH ratio)	7.3 U/ml	1.9 U/ml	3.74	30.8 %
NOX activity after addition of organic phase of 0.02 mM ferulic acid conversion products (product **10**, 4 vinylguaiacol,) (1:10 ferulic acid:NADH ratio)	8.2 U/ml	2.6 U/ml	3.13	9.4 %



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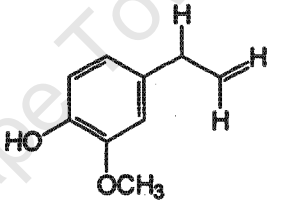

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Figure 4-21. Structures of the ferulic acid reaction products. 20 is a structure of one of the ferulic acid tetramers formed amongst others whose structures have not been elucidated. Its name is 3-carboxy-2'-(3-carboxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-5-yl)-(2-carboxyvinyl)-7,7'-dimethoxy-2,2',3,3'-tetrahydro-2,5'-bibenzofuran-3'-carboxylate. 10, is 4-vinyl guaiacol, the main product formed.

4.3.6 Significance and application of the novel NOX assay for antioxidant activity.

In a study by Orallo *et al.* (2002), it was shown that the vasorelaxant effect of resveratrol was caused by its inhibition on vascular NADH/NADPH oxidase activity. NOX hyperactivity has also been found to be responsible for a number of cardiovascular pathologies including artherosclerosis (Meyer and Schmitt, 2000). Excessively high NOX activity has been found to be responsible for the formation of tumours and cancers

(Cheuh, 2002), and the inhibition of tumour-associated NOX activity was found to inhibit tumour cell growth. Cancer cell lines have, as a result been found to be responsive to anti-NOX compounds such as retinol (Dai *et al.,* 1997).

In this study, the NOX assay was developed to aid identification of compounds that have an inhibitory effect on NOX activity and to correlate it with antioxidant activity measured by clinical methods. It was found that compounds that inhibited NOX activity were those that have been reported to have antioxidant activity and, thus, it is postulated here that there is a correlation. The hypothesis that anti-NOX activity can be correlated with antioxidant activity was supported by the results obtained in this thesis, as the antioxidant compounds synthesised in this study exhibited anti-NOX activity. Other antioxidants such as mycophenolic acid have also been associated with NOX inhibition (Park *et al.,* 2004).

The cellular antioxidant effects of the phenolic compounds synthesised in the course of this study may occur due to a number of mechanisms:
A) As directly operating antioxidants scavenging free radicals:

- The results obtained in the DPPH assays and the TEAC assays showed that the novel biocatalytically synthesised compounds can scavenge free radicals.
- At physiologically relevant concentrations (20 mM) NOX inhibition by aqueous products of a laccase- ferulic acid reaction was observed. These products were shown to be dimers and tetramers of ferulic acid and showed an inhibitory effect on NOX activity when added after the initiation of enzyme activity.

B) As indirectly acting antioxidants by:

- Protecting biomolecules such as NADH (Valdez *et al.,* 2002), and low density lipoproteins, as shown in the LDL oxidation experiments.
- Recycling oxidised molecules, such as recycling free cellular NAD^+ to NADH. NADH-dependent reduction of lipoic acid into its stronger antioxidant, reduced

form, dihydrolipoic acid, has been observed in erythrocytes (Haramaki *et al.*, 1997).

These functions are likely to be inter-related, as the protection is generally afforded by multiple mechanisms.

4.3.7 *In vivo* anti-NOX activity of antioxidants tested.

The link between antioxidants and NAD(P)H oxidase activity has been previously suggested in literature. Anti-NOX activity results in the decrease in ROS levels in vascular cells (ten Freyhaus *et al.*, 2006). This study has shown that compounds possessing anti-NOX activity were antioxidants. Many therapeutic strategies targeting NOX-derived ROS have been investigated, as a result of the experiments and clinical evidence of the role of ROS in vascular and endothelial dysfunction as well as Alzheimers dementia (Miller *et al.*, 2006). We propose that the compounds synthesized in this study having demonstrated anti-NOX activity could have applications in this area. The section below addresses specifically the potential *in vivo* effects of the antioxidants synthesised in this study.

The laccase –ferulic acid reaction products were shown to have NOX inhibitory effect. The inhibition of NOX results in the inhibition of the formation of NAD^+ from NADH as discussed previously and shown in the equation below.

$$2NADH + O_2 \xrightarrow{NOX} 2NAD^+ + 2H_2O$$

In cells, it is unlikely that the cytosolic concentration of NADH would be higher than phenolic concentrations; cytosolic concentrations of free NADH have been reported to be in the micromolar range (Sies, 1982), and most NOX assay methods are performed using micromolar concentrations of NADH as well (Orallo *et al.*, 2002; Dai *et al.*, 1997; Sun *et al.*, 2000). Methods used to test the effect of inhibitors have used an inhibitor ratio of 1:10 to 50 test inhibitor to NADH (Orallo *et al.*, 2002 and Sun *et al.*, 2000). In this

present study, assays used a 1:10 ratio, and the results shown previously were obtained (Figure 4-20, Table 4-8).

4.3.7.1 The *in vivo* application of the compounds with detected NOX inhibition ability in this study

The NOX assay designed in the course of this study can be used to test for antioxidant compounds. The antioxidant activity elicited by anti-NOX compounds has many possible mechanisms *in vivo*. These are listed below:

1. Directly acting antioxidants. Directly acting antioxidants have the ability to quench free radicals in themselves chemically. The antioxidants possessing antiradical activity such as compounds **39**, and **1**, would fall into this group.
2. Direct inhibition of NOX. Resveratrol has been found to have an inhibitive effect on NOX (Orallo *et al.*, 2002) which was also demonstrated in this study.
3. Inhibition of NOX activators. The flavonoids quercetin and rutin, as well as resveratrol, were found to inhibit NOX activity via the inhibition of protein kinase C, a NOX trigger (Deby-Dupont *et al.*, 2005; Slater *et al.*, 2003). Growth factors regulate NOX (Lessgue and Clempus, 2003), and VAS2870, a novel NOX inhibitor, was found to suppress NOX via platelet dependent growth factor BB (PDGF-BB). The ferulic acid polymers synthesized in this study possibly inhibit NOX via this mechanism, as they contain a derivative of the same functional group present in VAS2870.

It is significant that three of the most effective cardiovascular and cholesterol lowering HMG-CoA drugs directly inhibit vascular NOX (Wagner *et al.*, 2000). Therefore, the compounds synthesised biocatalytically in this study could potentially be used as drugs or drug precursors for therapy of cardiovascular disease. Increased antioxidant diets have been observed to have limited significance in clinical trials (Touyz, 2004). It is postulated that effective future therapy should involve antioxidants capable of inhibiting NOX activity, thereby inhibiting the generation of ROS, as opposed to scavenging ROS (Miller *et al.*, 2006). Several NOX inhibitors have been isolated including those described

previously and the major limiting factor of their usefulness is their non-selectivity. The NOX inhibitors such as DPI inhibit all flavin-containing enzymes including cytochrome P450 enzymes and are thus unsuitable for *in vivo* use (Miller *et al.*, 2006. The compounds synthesised in this study would therefore need investigation to ascertain their specificity.

4.3.8 Conclusions

The results obtained from the work described in this chapter demonstrate the antioxidant activity of the compounds produced from ferulic acid. Using the DPPH assay the laccase-catalysed reaction products were shown to possess anti-radical activity higher than their precursor, ferulic acid. The lipase transesterification products were also shown to have anti-radical activity using the TEAC and DPPH assays. Antioxidant synergy was also demonstrated for product **35** and ferulic acid. Therefore further antioxidant use of compound **35** would be improved by using it with ferulic acid, as demonstrated in this study.

The benefits of using a multi-test approach were considered and the polar paradox was illustrated by the antioxidant results of arbutin ferulate, **39**, which ranked highest in the LDL and TEAC assays but not in the DPPH assay. Therefore, compound **39** which has high antioxidant activity in lipophilic conditions should be used in lipophilic conditions, and compounds **20**, **33** and **35** in hydrophilic media as they were found to possess high activity in more hydrophilic media.

Interaction between NADH and phenolic compounds at high concentrations was shown and this interfered with the NOX assay. However, at concentrations that are physiologically applicable and at ratios applicable to cellular levels of NADH, inhibition of NOX was demonstrated by certain compounds.

The compounds found to possess antioxidant activity using other assays were found to possess anti-NOX activity. Specifically the ferulic acid polymeric products which were

shown to have higher antioxidant activity than their precursors in the DPPH assay also possessed higher activity as NOX inhibitors. This demonstrated a correlation between NOX inhibition with antioxidant activity. Other known antioxidants such as resveratrol, caffeic acids and resveratrol dimers were shown to have NOX inhibitory effects (Ting, 2004; Valdez *et al.*, 2002).

Factors that need further investigation and which are necessary to note during interpretation of results are:

1- The use of a bacterial NOX. Differences between mammalian and bacterial NOX responses will need to be investigated. It is significant that resveratrol which was found to inhibit mammalian NOX by Orallo *et al.* 2002 was also found to inhibit bacterial NOX in this study. This suggests possible similarity between mammalian and bacterial NOX inhibition. High sequence homology has been observed for bacterial NOX family members (Hendari *et al.*, 2004, Lountos *et al.*, 2004) but homology with mammalian genes would need to be investigated. The main advantage of using bacterial NOX would be that they are more readily available due to recombination technology.

2- Concentration effects. Higher or lower NOX or NADH concentrations than found in cells would make the assay unrepresentative. Physiological relevant ratios are needed. The problem is that there is neither a consensus on the cellular NADH levels (Howitz *et al.*, 2004) nor knowledge about cellular NOX levels. Further, several NOX isomers are thought to exist, exacerbating the problem (Dai *et al.*, 1997). A substrate: inhibitor ratio of 1:10 was used, as reported in previous studies (Dai *et al.*, 1997; Orallo *et al.*, 2002; Sun *et al.*, 2000). An investigation into the concentration effects of the various components of the assay namely; a) NOX enzyme, b) the substrate NADH and c) the putative inhibitors would be invaluable. Information on the bioavailability of the inhibitors would contribute significantly to establishing optimal concentrations of the different components the assay.

3- Once optimal ratios of substrates and enzyme are established, adaptation of the assay for high throughput analysis would be beneficial. This would involve the

scaling down of the 3 mL assay conditions used in this study, for application in standard 300µL 96 well ELISA plates.

4- Calculation of IC_{50} values for the inhibitors. The focus of the current study was to compare different antioxidant assays which were correlated with the NOX assay. In order to maintain consistency, similar antioxidant concentrations were used in all the assays to maintain uniformity. Future work will include the determination of IC_{50} values for the inhibitors tested, which are commonly used for enzyme inhibitors as used by Orallo *et al.*, 2002.
5- The absorbance of some phenolic antioxidants at 340 nm was problematic possibly; non-spectroscopic detection methods for the assay may be necessitated.

In conclusion, antioxidant assays used in this study demonstrated the antioxidant ability of biocatalytically synthesised compounds. The NOX assay which was investigated in this study has potential to be used as a more physiologically relevant *in vivo* antioxidant assay.

5 General conclusions

This dissertation describes the synthesis of potentially high-value products from ferulic acid using biocatalytic routes. The search for 'renewable' chemical feed-stocks that are 'natural' and cheap has led to the utilisation of agricultural waste. In this study, maize waste was used as a chemical feed-stocks using bioprocesses.

The compounds synthesised were shown to have antioxidant activity and antioxidants continue to have value as nutraceuticals and /or cosmeceuticals. The majority of this demand is met using artificially synthesised compounds such as butylhydroxytoluene (BHT) (Aucamp and Mitra, 2005). They are mainly used as stabilising agents for cosmetics or are used for protection against sunburn and aging. Different types of antioxidants are used in cosmetics and there is market proclivity for natural GRAS (generally regarded as safe) compounds. According to US and European legislature (US code of Federal regulations 1985 and the Council of the European Communities 1988), production of 'natural' substances includes those produced via enzymatic and microbial processes from natural precursors using biocatalysis and biotransformation. Thus, the compounds produced in this study would have applications as naturally produced compounds.

5.1 Applications of laccase synthesized derivatives of ferulic acid

To the best of our knowledge, the formation of 4-vinylguaiacol from the reaction of ferulic acid by laccase, has not been reported before. 4-Vinylguaiacol has been reported to be used for the synthesis of vanillin, and styrenes have been used as monomers for biodegradable plastics (Lee *et al.*, 1998; Iwabuchi *et al.*, 1983). 4-Vinylguaiacol is a flavour compound with a smoky, clove-like aroma and sells for US$3 500/KG (Newton, 2005).

The ferulic acid dimer (**23**) and tetramer (**20**) synthesized during the course of this study contain the highly bioactive benzo[b]furan functional group. Benzo[b]furans, a class of compounds which was previously classified as coumarans, have been found to possess diverse biological activities in many environments and has led to their applications in the pharmaceutical industry (McCallion, 1999). Benzo[b]furans have applications in the pharmaceutical industry as inhibitors of the enzymes acetylcholine acyltransferase, acetylcholine esterase, 5-lipoxygenase, monoamine oxidase, cyclooxygenase 2, aromatase, 5a-reductase and phosphodiesterase IV (Dell, 2001). Therefore, the ferulic acid polymers potentially have applications similar to the above as they possess the benzo[b]furan functional group.

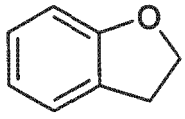


Figure 5-1. Structure of dihydrobenzofuran.

Benzofuran derivatives are also common antagonists of tachykinin (Horwell *et al.*, 1997) that are used for the treatment of disorders of the central nervous system and irritable bowl syndrome (Quartara and Altamura, 2006). Antithrombotic agents commonly contain the benzofuran nucleus (Ting *et al.*, 1997) as well as many other receptor antagonists (Dell, 2001).

The applications of benzofuran derivatives in industry have been extensively reviewed by Keay and Dibble (1996). Applications of carbofuran, a benzofuran in the agrochemical industry used as an insecticide is active against flies, mosquitos, aphids and larvae. Therefore, the biocatalytically synthesised compounds described in this thesis can potentially be used as insectisides or insectiside precursers. The same applies in their potential use in the dye and photographic material industry, polymer industry or pharmaceutical industry (Keay and Dibble, 1996). Of particular relevance to the work described in this thesis, the antioxidant activity of some benzofuran derivatives (hydroxybenzofurans) has been reported and was attributed to their medicinal

applications (Caldwell *et al.*, 1998; Keay and Dibble, 1996). Significantly, a series of 2,3-dihydrobenzofuran derivatives, with a hydroxyl and an alkoxy group (like OMe in the compounds synthesized in the course of this study, Fig 5-2) were evaluated as inhibitors of leukotriene biosynthesis (Hammond *et al.*, 1989). Recently, Grimm *et al.* (2006) reported the use of benzofurans as potent inhibitors of leukotriene biosynthesis for use as therapeutic drugs in the treatment of asthma and inflammatory disease. Several patents exist for compounds with the functional groups described, for example, a Japanese patent for preparation of 3-hydroxy-2,3-dihydrobenzofuran derivatives for the treatment of liver diseases (Kurokawa *et al.*, 1999) and for the treatment and prophylaxis of hepatitis C viral infections and associated diseases (Burns *et al.*, 2004).

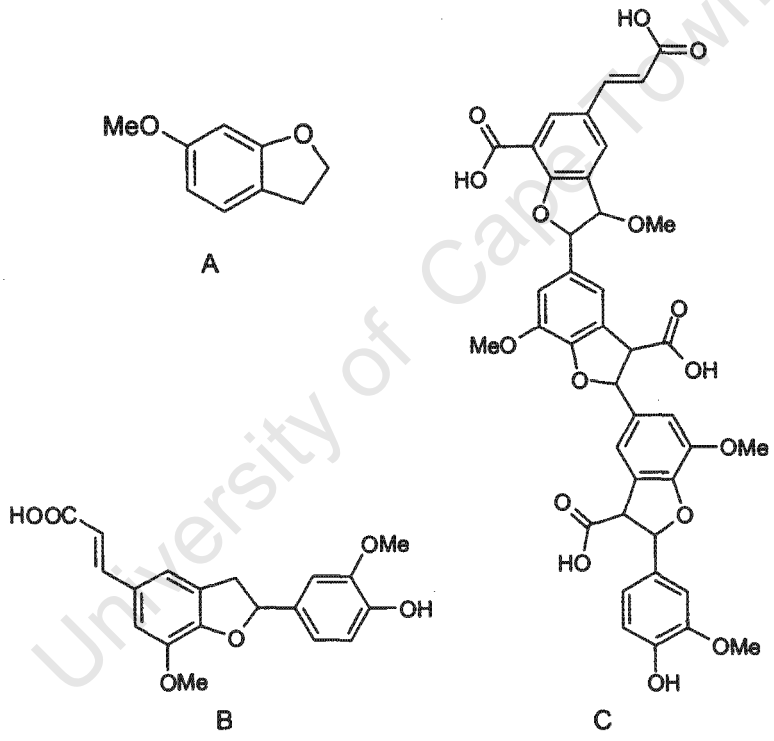


Figure 5-2. Structure of 2,3-dihydro-6-methoxy-benzofuran (A), a component of product 20 (C) and 23 (B).

The synthesis of benzofurans has achieved much attention due to the many applications the compounds possess. This is evidenced by the numerous publications on their synthesis which are reviewed by Friedrichsen (1996), Dell (2001), Horaguchi (1999), Kadieva and Oganesyan (1997), Hou *et al.* (2003) and reported by Harkal *et al.*, (2005).

The common chemical synthetic processes for the production of benzofurans involve the use of specialized metal catalysts such as palladium or copper acetylides for the coupling of halophenols with alkynes typically at 60°C (Dell, 2001). 2,3 Dihydrobenzofurans specifically, have been reported to be synthesized from the cycloaddition of styrenes, or stereoselectively by the oxidation of *p*-methoxy-substituted phenols with iodobenzene bis(trifluoracetate) in the presence of rich styrene derivatives. Abnormal Claisen rearrangements have also yielded 2,3-dihydrobenzofurans (reviewed by Friedrichsen, 1996). We provide here a method for the synthesis of benzofuran derivatives using biocatalysis.

The objective of this thesis was to biocatalytically synthesize bioactive compounds from ferulic acid, and it was accomplished by the synthesis of compounds possessing the highly bioactive dihydrobenzofuran functional group from the laccase reactions of ferulic acid. The 2,3-dihydrobenzofuran functional group in the ferulic acid dimer and tertramer synthesised in this study are present in a number of natural compounds and would therefore possess limited toxicity and allows their use as therapeutic compounds (Keay and Dibble, 1996; Dell, 2001). The biocatalytic production of these benzofuran derivatives provides a 'natural' route to their synthesis. In the future, compound **20**, which possesses three benzofuran functional groups, may be degraded by chemical or enzymatic means to yield the three benzofuran derivatives separately.

5.2 Application of lipase synthesized derivatives of ferulic acid

The use of lipases for the production of compounds similar in structure to the bioactive sterol ferulates was demonstrated. These compounds were found to possess some antioxidant activity, which for compound **35** was increased synergistically by the addition of 20% ferulic acid. Sterol ferulates have been demonstrated to possess activity against tumours (Akihisa *et al.*, 2000), lower blood cholesterol levels and can be used to treat nerve imbalance (Nakayama *et al.*, 1997). Their activity would need further *in vivo* investigation. The only *in vivo* analysis of the activity of these compounds (only paper found from a database search on Sci-finder and Sciencedirect) demonstrated the

inhibition of liver and serum hypercholesterolemia in rats fed high levels of lard (Suh *et al.*, 2005). Therefore, the sterol ferulates synthesized using lipases would need *in vivo* testing, and could potentially have applications as therapeutic drugs for the treatments of hypercholesterolemia and other diseases. The use of the sterol ferulates produced in this study will be used *in vivo* in further studies to gain insight into the mechanisms of the medicinal properties reported for these compounds.

This thesis describes a simple novel biocatalytic method for the production of sterol ferulates that are reported to possess many therapeutic properties. It describes a natural method for their synthesis and is the first such process described, to the best of the author's knowledge. Other published methods use extraction methods to source of these compounds from plant oils. This could potentially increase the use of these compounds as nutraceuticals.

Compound **39**, arbutin ferulate, was found to possess good antioxidant activity in a hydrophilic medium, better than its precursor ferulic acid. Further *in vivo* studies using melanocyte cell lines, will investigate a possible link between melanogenesis inhibition and antioxidant activity. This will be possible as arbutin is a melanogenesis inhibitor and its chemical coupling with an antioxidant, could potentially yield interesting *in vivo* effects possibly with therapeutic applications.

5.3 Significance of NOX assay

The need for *in vivo,* biologically relevant assays, lead to the development of the novel anti-NOX assay. NADH oxidase is a ubiquitous enzyme and is involved in cell redox systems (Mathews and van Holde, 1990). Its activity has been found to have an effect on the oxidative status of cells (Bolotin *et al.*, 2001; De Angelis and Gobbetti, 2004) and hence its antioxidative abilities. There was anti-NOX activity noted by most of the antioxidants tested and a significant effect was observed for those which were biocatalytically synthesised.

ROS have been shown to be involved in many diseases. NAD(P)H oxidases, which produce ROS, are as a result involved in the pathology of many diseases. Specifically, NOX have been shown to be the predominant ROS producers in the vascular wall and are thus implicated in the pathophysiology of cardiovascular disease (Lessegue and Clempus, 2003). NOX have also been implicated in the pathogenesis of cancers (Suh *et al.*, 1999) and Alzheimer's disease (Shimohama *et al* ., 2000). As a result, anti-NOX compounds potentially possess significant therapeutic roles. Inhibition of NOX was demonstrated in this study by the biocatalytically synthesised ferulic acid polymers.

Findings in this study supported the hypothesis that anti-NOX activity was linked to antioxidant activity. The recent findings by ten Freyhaus (2006), supports this hypothesis also, as the novel compound, VAS2870, they described to possess anti-NOX activity, was shown to suppress ROS liberation in vascular smooth muscle cells. The ferulic acid dimers and tetramers synthesized in this study were shown to possess antioxidant activity as well as anti-NOX activity. The anti-NOX activity they possess therefore has potential applications in the treatment of cardiovascular disease. It is noteworthy that the novel compound described by ten Freyhaus (2006) contains a nitrogen substituted benzofuran (benzoxazoles) functional group (Fig 5-3).

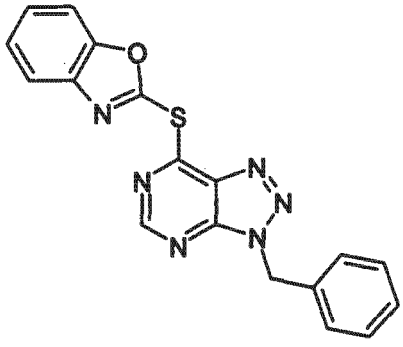


Figure 5-3. Structure of VAS2870, a novel NOX inhibitor synthesized by ten Freyhaus *et al.*, 2006.

The anti-NOX assay developed in this study is easy to perform and can be adapted for high throughput analysis of putative NOX inhibitors, using microtitre plates. This will be useful in drug design where combinatorial chemistry produces large chemical libraries, for use as drug targets. The use of a bacterial NOX obtained by heterologous protein expression makes the NOX readily available for use at large scale. This assay potentially provides an *in vivo* assay of antioxidant activity. NOX activity has been shown to be suppressed via antioxidative means, and the correlation has been established in *in vivo* analysis (ten Freyhaus, 2006; Dai *et al.*, 1997).

Many factors will need to be investigated before the NOX inhibitors described in this study can be used therapeutically including the specificity of the inhibitors against the different NOX isozymes, as well as other cellular enzymes. A direct relationship will need to be established between *in vivo* NOX inhibition and inhibition of the bacterial NOX used in this study. The toxicity of the compounds synthesized in this study should be minimal as they were derived from naturally occurring dietary ferulic acid. Also the market preference for "naturally" produced pharmaceuticals will broaden their relevance.

In conclusion, this thesis describes the development of novel biocatalytic processes for the synthesis of bioactive compounds from ferulic acid. Most of these compounds had higher antioxidant activity than their precursor ferulic acid, thereby adding value to ferulic acid. The production of ferulic acid tetramers has not been reported before and the biocatalytic production of sterol ferulates similar to those found in γ-oryaznol is described in this thesis. The potential applications of the compounds produced in this study as they posses bioactivity are numerous.

Appendix

SDS-PAGE

10% Resolving Gel

13.35 mL 30 % Acrylamide Stock

15.00 mL Tris-HCl Buffer (pH 8.8)

9.25 µL dH_2O

400 µL 10% SDS (Replace with dH_2O if making non-denaturing gel)

300 µL fresh 10% APS

20 µL TEMED

4 % Stacking gel

2 mL 30 % Acrylamide stock

1.9 mL Tris-HCl buffer (pH 6.8)

9.25 mL dH_2O

mL 80% glycerol

100 µL fresh 10 % APS

150 µL 10% SDS

20 µL TEMED

2X Dissociation Buffer

5.0g SDS

2.0 mL Mercaptoethanol

7.5 mL Glycerol

2,5 mL 0.2% bromophenol blue

6.3 mL 1M Tris-HCl buffer (pH 6.8)

28.7 mL dH_2O

30% Acrylamide stock

75 g Acrylamide

2 g Bis-Acrylamide

Dissolve in 250 mL dH_2O

Coomassie Protein Staining solution

450 mL Methanol

100 mL Glacial Acetic acid

100 mL Glycerol

380 mL dH_2O

Bradfords reagent

20 mg Coomassie G

10 ml 95% Ethanol

20 ml 85% Phosphoric acid (H_3PO_4)

Media

Trametes defined media (TDM)

Glucose	20 g
Peptone	10.46 g
KH_2PO_4	4 g
$MgSO_4$	1.0 g
$CaCl_2$	0.2 g
NaCl	0.5 8
Trace elements	20 mL

Make up to 2 L in water.

100X Trace Elements

Iron sulphate	0.28 g
Copper sulphate	0.016 g
Zinc chloride	0.034 g
Manganese sulphate	0.169 g
Cobalt chloride	0.095 g
Nickel chloride	0.0012 g
Ammonium molybdate	0.309 g

Make up to 500 mL in water.

10 x PBS

80 g/L NaCl

2 g /L KCl

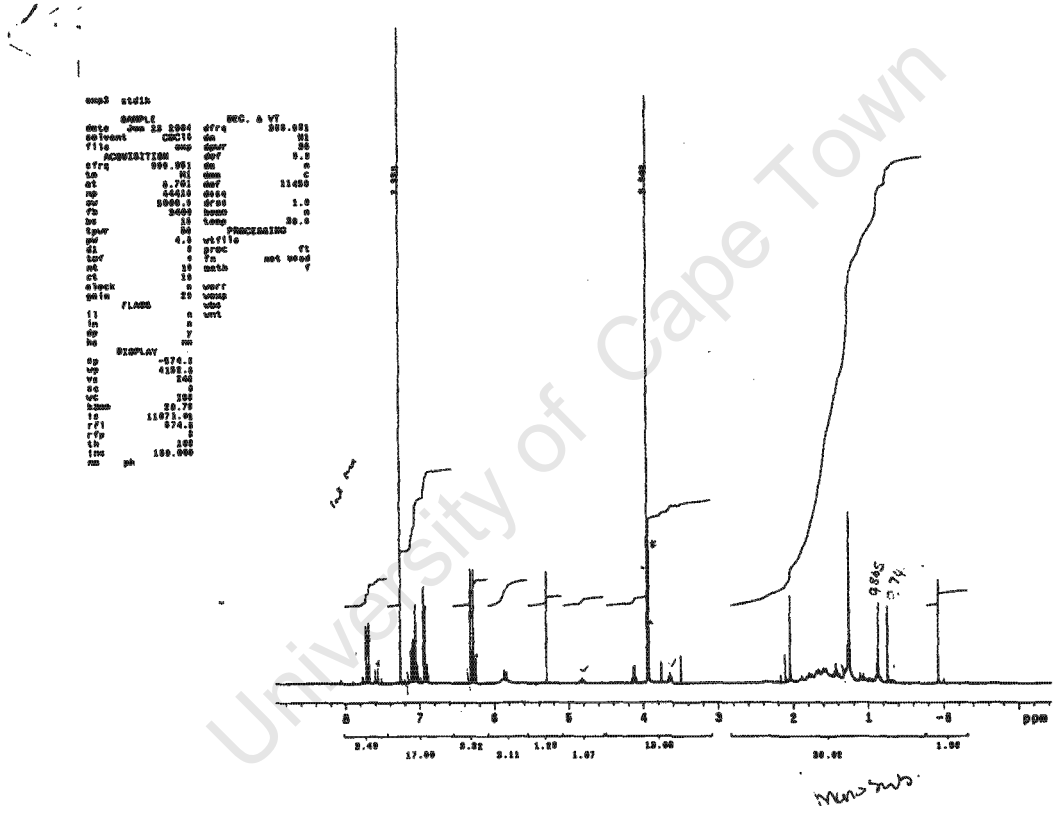
26.8 g/L Na_2HPO_4-$7H_2O$

204 g/L $KHPO_4$

Adjust pH to 7.4 with HCl

Sterilize by autoclaving

NMR of 1:4 mixture of 1 and 35



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