OXIDATIVE COUPLING OF DIBENZYL BUTANOLIDES
CATALYZED BY PLANT CELL CULTURE EXTRACTS

By

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(Chemistry)

We accept this thesis as conforming
to the required standard

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The University of British Columbia
Vancouver, Canada

Date June 10, 1990

DE-6 (2/88)
This thesis aims to develop a new and inexpensive synthetic route to the anti-cancer drug etoposide (6) via 4’-demethylpodophyllotoxin (4) or 4’-demethylepipodophyllotoxin (5) involving the oxidative coupling of a dibenzylbutanolide catalyzed by a cell-free extract (CFE) from plant cell culture.

This step was studied in depth using the Catharanthus roseus CFE-catalyzed biotransformation of trans-2-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3-hydroxy-4-methoxybenzyl)butanolide (58) to 1-(3,5-dimethoxy-4-hydroxyphenyl)-6-hydroxy-3-hydroxymethyl-7-methoxy-1,2,3,4-tetrahydro-2-naphthoic acid γ lactone (59) as a model. The optimum values of reaction pH, enzyme:substrate ratio and cofactor:substrate ratio were determined. The butanolide 58 was synthesized by a route involving the Stobbe condensation of 3-benzyloxy-4-methoxybenzaldehyde with dimethylsuccinate to yield 2-(3-benzyloxy-4-methoxybenzylidene)butanedioic acid 1-methyl ester (69). Hydrogenation of 69 to 2-(3-benzyloxy-4-methoxybenzyl)butanedioic acid 1-methyl ester (70) followed by reductive lactonization afforded 3-(3-benzyloxy-4-methoxybenzyl)butanolide (71). Alkylation of 71 with 4-benzyloxy-α-bromo-3,5-dimethoxytoluene (72) gave trans-2-(4-benzyloxy-3,5-dimethoxybenzyl)-3-(3-benzyloxy-4-methoxybenzyl)butanolide (73) which was then converted to the butanolide 58 by catalytic hydrogenolysis.

In order to investigate the effect of different aromatic substituents on the oxidative coupling of butanolides, C. roseus CFE-catalyzed biotransformations of trans-2-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-methylenedioxybenzyl)butanolide (74) and trans-2-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxy-α-hydroxybenzyl)butanolide (94) were also performed. The biotransformation of 74 gave 2-(3,5-dimethoxy-4-hydroxybenzylidene)-3-(3,4-methylenedioxybenzyl)butanolide (76) as the sole isolated product. A pathway involving oxidative demethylation is proposed to account for the balance of the unrecovered material.

The butanolide 94, a potential precursor to etoposide, was prepared from piperonal. The lithium anion of 1-bis(phenylthio)methyl-3,4-methylenedioxybenzene (97) and the bromide 72 were added consecutively to but-2-en-4-olide to afford trans-2-(4-benzyloxy-3,5-dimethoxybenzyl)-3-(3,4-methylenedioxy-α,α-bis(phenylthio)benzyl)butanolide (96). A synthetic sequence involving the
oxidation of 96 to trans-2-(4-benzyloxy-3,5-dimethoxybenzyl)-3-(3,4-methylenedioxybenzoyl)-
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groups gave the catechol 94. Unfortunately, the CFE-catalyzed oxidation of 94, following treatment
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4: \( R = H; R' = OH \)
5: \( R = OH; R' = H \)
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Finally, I am deeply grateful to my parents for their patient support.
The following is a list of the abbreviations used throughout this thesis.

Ac = acetyl
APT = attached proton test
Ar = 3,4,5-trimethoxyphenyl
aq. = aqueous
br = broad
Bu = butyl
C. = Catharanthus
CFE = cell-free extract
COSY = correlated spectroscopy
d = doublet
DCC = dicyclohexylcarbodiimide
DCI = desorption chemical ionization
dd = doublet of doublets
ddd = doublet of doublets of doublets
DNA = deoxyribonucleic acid
El = electron impact ionization
Et = ethyl
EtOAc = ethyl acetate
FAB = fast atom bombardment ionization
HPLC = high-pressure liquid chromatography
HRP = horseradish peroxidase
IR = infrared
m = multiplet
Me = methyl
MS = mass spectrum
LDA = lithium diisopropylamide
M+ = molecular ion
Mp = melting point
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<td>NMR</td>
<td>nuclear magnetic resonance (one-dimensional unless otherwise specified)</td>
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<tr>
<td>P.</td>
<td><em>Podophyllum</em></td>
</tr>
<tr>
<td>PCC</td>
<td>pyridinium chlorochromate</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>p-Ts</td>
<td><em>para</em>-toluenesulfonyl</td>
</tr>
<tr>
<td>Rf</td>
<td>retention factor</td>
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<tr>
<td>s</td>
<td>singlet</td>
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<tr>
<td>sp.</td>
<td>species</td>
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<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>THP</td>
<td>tetrahydropyranyl</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
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1. INTRODUCTION

1.1. PODOPHYLLOTOXINS

1.1.1. CHEMISTRY AND PHARMACOLOGY

Podophyllotoxin (1)\(^1\)\(^,\)\(^2\) is an interesting member of the naturally-occurring family of compounds known as lignans which are derived biosynthetically from the shikimate pathway\(^3\)\(^-\)\(^5\). Podophyllotoxin has been the object of much study as it presents a challenging synthetic target and, more importantly, has proven anti-cancer properties.

Podophyllotoxin is isolated commercially from the American mandrake (\textit{Podophyllum peltatum} Linnaeus) and the related Indian species \textit{P. emodi} Wallich (\textit{P. hexandrum} Royle). The dried roots and rhizomes, known as podophyllum, are extracted with alcohol to produce a resin (podophyllin) containing podophyllotoxin. For example, podophyllum from \textit{P. peltatum} yields 4-6\% podophyllin\(^6\) which affords 9-10\% podophyllotoxin upon chromatographic separation\(^7\). Although the medicinal properties of \textit{P. peltatum} had long been known by North American natives, it was not until 1942 that Kaplan\(^8\) demonstrated the efficacy of podophyllin in the treatment of venereal warts. This finding sparked much interest in the biological and chemical properties of podophyllin and its constituents and this has continued until the present.

The main structural feature of the podophyllotoxins 1-6 is their high degree of rigidity. This results from the fact that C(1) and C(4) are fixed in the plane of ring B while being held in a \textit{trans} orientation about the lactone ring (Fig. 1). Examination of a Dreiding model also suggests that podophyllotoxin exists in a single conformation in which ring E is virtually fixed axially. This highly-strained structure presents considerable synthetic difficulties owing to its facile epimerization to the much more stable isomer picropodophyllin (7, Scheme 1), which does not inhibit cell division. Dreiding models of picropodophyllin indicate two conformations and an essentially complete freedom of rotation of ring E in a quasi-equatorial orientation. The relative stability of picropodophyllin is also demonstrated by an equilibrium constant of 37.0 at 31\°\,C\(^9\) for the epimerization shown in Scheme 1.

Podophyllotoxin acts as an anti-cancer drug by preventing microtubule assembly and thus separation of the chromosomes during mitosis. Although the toxic side-effects of podophyllotoxin
Podophyllotoxin 1: $R = H; R' = OH; R'' = CH_3$

Epipodophyllotoxin 2: $R = OH; R' = H; R'' = CH_3$

Deoxypodophyllotoxin 3: $R = R' = H; R'' = CH_3$

4'-Demethylpodophyllotoxin 4: $R = H; R' = OH; R'' = H$

4'-Demethylepipodophyllotoxin 5: $R = OH; R' = H; R'' = H$

Etoposide 6: $R = CH_3$; $R' = H; R'' = H$

Fig. 1. Podophyllotoxin (1).
such as nausea, vomiting and bone marrow damage severely limit its use as a chemotherapeutic agent, it is employed externally in the form of podophyllin for the treatment of benign skin growths\textsuperscript{10}.

It has been found that certain glycosides of 4'-demethylepipodophyllotoxin (5) are also effective anti-cancer drugs but lack the unacceptable side-effects of podophyllotoxin\textsuperscript{11}. The most important compound of this class is etoposide (6)\textsuperscript{12}, which is used clinically in the treatment of myelocytic leukemia, neuroblastoma, bladder cancer, testicular cancer and small-cell lung cancer\textsuperscript{13}. Unlike podophyllotoxin, these compounds do not inhibit microtubule assembly; they are believed to derive their anticancer properties from interference with DNA topoisomerase II, an enzyme which makes reversible double-stranded breaks in DNA in order to prevent the tangling resulting from DNA replication\textsuperscript{14}. Recent work has suggested that the role of etoposide is to promote both DNA cleavage and inhibit its subsequent religation\textsuperscript{11,15}. The presence of a hydroxyl group at C(4')\textsuperscript{16} and a β orientation of the C(4) substituent have been shown to be critical for such anti-tumor activity. The actual nature of the C(4) substituent is much less important\textsuperscript{17}. The relative abilities of various podophyllotoxin derivatives to inhibit tubulin polymerization and DNA topoisomerase II are summarized in Table 1.
TABLE 1
INHIBITION OF TUBULIN POLYMERIZATION AND DNA TOPOISOMERASE II BY PODOPHYLLOTOXIN DERIVATIVES\textsuperscript{17,18}

<table>
<thead>
<tr>
<th>Compound</th>
<th>ID\textsubscript{50} for Tubulin Inhibition (\mu M)</th>
<th>Relative Topoisomerase Inhibition (%)</th>
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<tbody>
<tr>
<td>Podophyllotoxin (1)</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>Picropodophyllotoxin (7)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Epipodophyllotoxin (2)</td>
<td>5</td>
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<td>Deoxypodophyllotoxin (3)</td>
<td>0.5</td>
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</tr>
<tr>
<td>4'-Demethyl/podophyllotoxin (4)</td>
<td>0.5</td>
<td>0</td>
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<td>4'-Demethylepipodophyllotoxin (5)</td>
<td>2</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Etoposide (6)</td>
<td>no inhibition at 100 \mu M</td>
<td>75</td>
</tr>
</tbody>
</table>

1.1.2. SYNTHESIS

The first total synthesis of podophyllotoxin, performed by Gensler et al., is shown in Scheme 2\textsuperscript{19,20}. The \textit{cis}-1,2-\textit{trans}-2,3 stereochemistry was obtained by first converting the intermediate (\pm)-\alpha-apopicropodophyllin ((\pm)-18) to (-)-picropodophyllin (7). Saponification of (\pm)-18 with sodium hydroxide gave (\pm)-\alpha-apopicropodophyllic acid ((\pm)-(19)), which was resolved with quinine. Lactonization and dehydration of the \textit{levo} enantiomer with sulfuric acid to afford (-)-18 was followed by treatment with hydrogen chloride in dilute acetic acid and basic hydrolysis to yield (-)-picropodophyllin. The enolate of 4-tetrahydropicropodophyllin (20) was then prepared using triphenylmethyl sodium: the addition of excess acetic acid at room temperature resulted in an irreversible kinetically-controlled protonation
Scheme 2.

1) NaOH  
2) Separation

Ar = 3,4,5-trimethoxyphenyl
Scheme 2 (continued).
which, following hydrolysis of the acetal moiety, afforded a 9:11 mixture of podophyllotoxin and picropodophyllin. Although the length of this procedure does not make it an efficient synthetic route to podophyllotoxin, the kinetic protonation method used to produce the cis-1,2 stereochemistry has since been employed in numerous other syntheses of podophyllotoxin\textsuperscript{21-24}.  

Scheme 2 (continued).
Recently, several total syntheses of podophyllotoxin\textsuperscript{25-30} and epipodophyllotoxin\textsuperscript{31} have been published which achieve the required stereochemistry in a much more selective manner. One of the most practical is the route devised by Rodrigo (Scheme 3)\textsuperscript{25}. 3,4,5-Trimethoxybenzaldehyde was added to the lithiated dimethyl acetal of 6-bromopiperonal (22) to afford the alcohol 23\textsuperscript{32,33} which was subjected to a Diels-Alder reaction with dimethyl acetylenedicarboxylate in the presence of a catalytic amount of acetic acid. Subsequent hydrogenation yielded the endo ester 25 which was epimerized to the \textit{trans} diester 26 with sodium methoxide. Lithium triethylborohydride then selectively reduced the C(3) ester to the alcohol 27. The 1,4-oxygen bridge was cleaved by hydrogenolysis with Raney nickel and the resulting diol 28 converted to the acetonide 29. Saponification of the methyl ester proceeded without inversion at C(2) to give the ketal 30 which, upon treatment with very dilute acid, afforded neopodophyllotoxin (31), which has the correct stereochemistry about ring C but employs the C(11), rather than the C(4), hydroxyl to form the lactone ring. Neopodophyllotoxin has been converted previously to podophyllotoxin\textsuperscript{34} by saponification of the lactone followed by re-esterification with dicyclohexylcarbodiimide. This 12-step route produced (±)-podophyllotoxin in an overall yield of 9.4% and did not require any chromatographic separations.

Asymmetric syntheses of (-)-podophyllotoxin\textsuperscript{23} and (-)-deoxypodophyllotoxin \textsuperscript{235} have been reported. (-)-Deoxypodophyllotoxin has been converted synthetically to (-)-podophyllotoxin\textsuperscript{36}.

The synthesis of the etoposide precursor 4'-demethylepipodophyllotoxin (5) via podophyllotoxin as performed by Kende \textit{et al.}\textsuperscript{22} is shown in Scheme 4. This synthesis employs Gensler’s kinetic protonation strategy to achieve the correct \textit{cis}-1,2-\textit{trans}-2,3 stereochemistry but uses a different method to arrive at the the picropodophyllin intermediate. Piperonal (32) was treated with the Wittig reagent Ph\textsubscript{3}P+CH\textsubscript{3}Br\textsuperscript{-} and then with bromine in methanol to afford the methoxy dibromide 33. Addition of diethyl 3,4,5-trimethoxybenzylidenemalonate to the lithiated dibromide 33 gave a diastereomeric mixture of the aryltetralin diesters 34. Subsequent cleavage of the ether moiety was followed by oxidation of the resultant benzylic alcohol to the keto diester 35, hydrolysis and decarboxylation to give the \textit{trans} keto acid 36. Picropodophyllone (38) was then prepared from 36 by hydroxymethylation at C(3) with formaldehyde followed by a retro-aldol thermolysis. Picropodophyllone was reduced to picropodophyllin with lithium aluminum tri-i-butoxy hydride and converted to the silyl ether 39 which was
Scheme 3.
Scheme 3 (continued).
Scheme 4.
treated with lithium diisopropylamide to produce the corresponding enolate. Kinetically-controlled protonation with pyridinium hydrochloride afforded a mixture of 39 and the silyl ether of podophyllotoxin (40, 48% yield after one recycle of residual 39). 4'-Demethylepipodophyllotoxin (5) was obtained from 40 by treatment with anhydrous hydrogen bromide followed by hydrolysis. The synthesis of 5 was thus accomplished in 13 steps in 2.4% overall yield.
The preparation of etoposide from podophyllotoxin is shown in Scheme 5. Podophyllotoxin was treated successively with hydrogen bromide and aqueous barium carbonate to afford the 4'-demethyllepipodophyllotoxin (5). The C(4') hydroxyl was protected with benzyl chloroformate and the resultant carbonate reacted with 2,3,4,6-tetraacetyl-β-D-glucopyranose in the presence of boron trifluoride etherate to yield the glycoside. This same product was obtained when epipodophyllotoxin was replaced with podophyllotoxin: the C(4) hydroxyl stereochemistry of the aglycone is therefore not important in the synthesis of etoposide. The acetyl groups were removed with zinc acetate in methanol, the carbonate moiety was cleaved by catalytic hydrogenolysis and the resultant glycoside was condensed with acetaldehyde to yield etoposide.

Even though the syntheses of podophyllotoxin shown in Schemes 3 and 4 are among the most efficient and direct available, they are still not suitable for a large-scale (e.g. 10^2-10^3 kg) industrial preparation of the product (and hence etoposide) as all are multi-step operations employing expensive reagents and techniques which are practical only in the laboratory. Transformations involving microbial and plant cell cultures, however, do not share these problems and may thus offer an inexpensive route to the preparation of etoposide via podophyllotoxin or an intermediate such as 4 or 5. The principal advantage of microbial and plant cell cultures is their ability to convert starting material to product in a "one-pot" biosynthetic operation using simple precursors. Furthermore, the difficulties involved in scaling up such a process to an industrial level of production are minimal in comparison with those of the synthetic approach.

An example is the recently-reported microbial transformation of (-)-deoxypodophyllotoxin (3) to (-)-epipodophyllotoxin (2) by a species of *Penicillium* in 100% yield. (-)-Deoxypodophyllotoxin was obtained from the seeds of *Hernandia ovigera*, where it constitutes 2.4% of the dry weight. The success of this approach suggested that a biomimetic route to podophyllotoxin, or one of its derivatives, employing both synthetic and biological transformations might repay investigation. We wished to determine if plant cell cultures in particular could be exploited in such a route as much work has been performed in this laboratory on both the plant cell culture-mediated synthesis of 3',4'-anhydrovinblastine and the biosynthesis of podophyllotoxin in *P. peitatum*. 
Scheme 5.

1) HBr
2) BaCO₃, H₂O

ClCO₂CH₂Ph
pyridine

BF₃·OEt₂
Scheme 5 (continued).
It has long been known that many valuable pharmaceutical, flavor and perfume compounds, which would present a major challenge to the synthetic chemist, can be isolated from plants. Such isolation procedures are usually lengthy, however, as they involve the purification of a small amount of product from a very large mass of differentiated plant tissue. Consequently, the final product must be very costly if such a process is to be economically viable. Considerable effort has thus been spent in replacing the normal plant with a culture of undifferentiated cell tissue in an attempt to increase the yields and the efficiency of the isolation step. To prepare such a culture, plant cells are transferred from root tissue, for example, to either a solution or a solid medium where they continue to grow and divide but do not evolve into separate tissues. Growth is rapid and unaffected by environmental variables as it occurs under optimized laboratory conditions. Growth regulators may be introduced to promote the formation of a particular product as well as culture proliferation. Unfortunately, whole cell cultures have enjoyed very limited success to date owing to poor large-scale productivity and genetic\textsuperscript{50} in the form of an increased number of chromosomal and gene mutations\textsuperscript{51}. In addition, the undifferentiated cells of the culture might not produce a target compound normally found in a distinct tissue of the intact plant.

The potential of plant cell cultures has been demonstrated by the production of the cardiac drug ubiquinone-10 by suspension cultures of \textit{Nicotiana tabacum} in amounts ten times greater than those found in the intact plant\textsuperscript{52}. In fact, the naphthoquinone shikonin is currently being produced commercially by cultures of \textit{Lithospermum erythrorhizon}\textsuperscript{53-55}. By contrast, callus tissue culture (cells arising by multiplication of plant organ segments and grown on a solid medium) of \textit{P. peltatum} only afforded podophyllotoxin in yields\textsuperscript{56} similar to those of the intact plant\textsuperscript{6,7}.

Problems with whole cell transformations usually arise from the fact that the conversion of starting material to product is being carried out by a complex biological system rather than by simple laboratory reagents. The introduced starting material therefore usually represents an advanced, rather than early, stage of biosynthesis of the product in order to minimize diversion of starting material into competing reaction pathways. In addition, the selectivity of the cell wall may prevent the starting material from diffusing into the cells. Finally, isolation of product will still be difficult if the product remains inside the
cell because the entire biomass must be extracted, which again results in a complex mixture of product and undesired cell material. Despite these obstacles, this methodology holds much promise as shown by the semi-continuous conversion of β-methyldigitoxin to the cardiac drug β-methyldigoxin by *Digitalis lanata* in 80% yield\(^5\,^7,\,^8\).

One solution to these problems is to replace the whole living cells with a cell-free extract (CFE) (Scheme 6), which can be considered as a solution of enzymes. Ideally, this purification is limited to a removal of buffer-insoluble material, such as the cell wall, but it may also include chromatography to isolate the required enzymes. The enzymes of interest are usually characterized by their concentration (or activity, expressed in units per millilitre) and purity (or specific activity, expressed in units per milligram of soluble protein). One unit is defined as the amount of enzyme required to convert 1 micromole of substrate to product in 1 min at 25° C under the ideal measuring conditions.

Isolation of product is greatly facilitated because only a small fraction of the cell culture biomass is used for the conversion (referred to as a biotransformation in this case since the starting material is not

---

**Cell Culture or Plant Tissue**

1. Filtration
2. Homogenization with Buffer (pH\(_r\))
3. Centrifugation

Pellet __Supernatant (CFE)__

---

Substrate __Co-factors__

CFE __pH\(_r\)__ __Extraction__ __Product__

Scheme 6.
prepared *in situ*), which is performed as if it were a conventional synthetic process with the CFE acting as a reagent. The CFE actually acts as a catalyst in the reaction but since it is invariably inactivated during product isolation (usually by the organic solvents used in the subsequent extraction) it cannot strictly be referred to as such. The reaction conditions can be precisely determined and a wide variety of substra-

Scheme 7.
tes examined because the reaction is carried out in a homogeneous solution rather than inside a cell. It is evident that the starting material and any necessary co-factors for the reaction must be supplied directly to the CFE mixture as these can no longer be synthesized in situ. This requirement is a major consideration for a potential commercial process: for example, biotransformations employing costly nicotinamide adenine dinucleotide phosphate (NADPH), a co-factor of the selective oxidizing enzyme cytochrome P-450, would be practical only if a particularly expensive final product was involved.

Other drawbacks of this approach include the possibility of losing the enzyme or its activity during CFE preparation and the increased complexity of the overall procedure which does not make it as suitable for industrial-scale operations as whole cell-catalyzed transformations. However, the latter problem could be partly avoided by using the CFE enzymes in a purified and immobilized form which would permit their recovery and re-use after each biotransformation.

An illustration of such a biotransformation is the preparation of 3',4'-anhydrovinblastine 47, an immediate precursor to the clinical anticancer drug vinblastine, using Catharanthus roseus cell culture. Incubation of catharanthine (45) and vindoline (46) with C. roseus CFE in the presence of hydrogen peroxide gave a 25% yield of 3',4'-anhydrovinblastine. (Scheme 7)48. This value increased to 38% when immobilized enzymes from the same culture were employed44.

1.3. BIOSYNTHESIS OF PODOPHYLLOTOXIN

A preparation of podophyllotoxin using plant cell cultures clearly requires an understanding of the biosynthetic pathway involved. The sequence proposed by Dewick59 is shown in Scheme 8. Phenylalanine (48) is converted via cinnamic acid (49) in several steps to ferulic acid (50) which undergoes oxidative coupling to the hypothetical bis(quinonemethide) 51. Subsequent steps lead to matairesinol (52) and yatein (53). The cyclization of yatein to deoxypodophyllotoxin (3) is followed by hydroxylation at C(4) to give podophyllotoxin. This hypothesis is based on feeding experiments with Podophyllum sp. which showed the incorporation of radioactively-labelled 48, 49, 50, 52, 53 and 359-62 into podophyllotoxin.
Scheme 8.
Scheme 8 (continued).

Scheme 9.
The mechanism of the cyclization of yatein to deoxypodophyllotoxin is particularly interesting on account of the stereochemistry of the product. Dewick has suggested that in a second oxidative coupling step yatein is oxidized to the quinone methide 54 which then undergoes nucleophilic attack by ring B to form the aryltetralin skeleton. A serious objection to this mechanism is that it does not explain why the cis-1,2 isomer deoxypodophyllotoxin (3) would be produced in preference to the much more stable trans-1,2 isomer deoxyisopodophyllotoxin (55). This preference is demonstrated by the oxidation of yatein with thallium(III) oxide (Scheme 9) which gave a 60% yield of 55 in a reaction proceeding through the same intermediate 54: no deoxypodophyllotoxin was isolated. However, there may be further intermediates in the pathway between yatein and deoxypodophyllotoxin which have not yet been identified.

The possibility of a quinone methide intermediate in this cyclization suggests the involvement of peroxidases, enzymes which are known to catalyze the formation of such species. For example, the quinone methide 57 was identified as the product of the oxidation of eugenol (56) by horseradish peroxidase (HRP) as shown in Scheme 10. Furthermore, at least one form of peroxidase (lignin peroxidase) is capable of oxidizing not only aromatic compounds bearing solely alkyl and methoxy groups but even unsubstituted aromatic systems such as pyrene: the absence of a para hydroxyl group, as in eugenol, thus need not present an obstacle to the formation of a quinone methide such as 54.
1.4. PEROXIDASES

Peroxidases are iron hemoproteins which act as one-electron oxidizing agents for a wide variety of organic and inorganic substrates. Most peroxidases, including all plant-derived species, have been found to contain the prosthetic group ferriprotoporphyrin IX. Their principal biological role in plants is likely the removal of harmful hydrogen peroxide from the cell via the polymerization of phenolic compounds by oxidative coupling. Peroxidases are a very attractive class of enzymes for CFE-catalyzed biotransformations because they are very stable, have a well-known mode of action and use inexpensive hydrogen peroxide as a co-factor.

The mechanism of peroxidase action at low concentrations of hydrogen peroxide ($10^{-6}$-$10^{-4}$ M) is shown in equations (1)-(4). By convention, the oxidized forms of peroxidase are referred to as compounds I - III.

\[
\text{native enzyme} + \text{H}_2\text{O}_2 \rightarrow \text{compound I} + \text{H}_2\text{O} \quad (1)
\]

\[
\text{compound I} + \text{AH} \rightarrow \text{compound II} + \text{A}^- \quad (2)
\]

\[
\text{compound II} + \text{AH} \rightarrow \text{native enzyme} + \text{A}^- \quad (3)
\]

\[
2\text{A}^- \rightarrow \text{products} \quad (4)
\]

The native (ferric) enzyme first undergoes a two-electron oxidation with a peroxide to form the intermediate compound I (equation (1)). Several peroxides ($\text{H}_2\text{O}_2$, $\text{C}_2\text{H}_5\text{OOH}$, $\text{CH}_3\text{OOH}$, $\text{CH}_3\text{CO}_3\text{H}$) can serve as electron acceptors but hydrogen peroxide is by far the most effective. Interestingly, various halogen oxides such as $\text{HOCl}$, $\text{HOBr}$ and $\text{ClO}_2$ can also replace hydrogen peroxide: in fact, the HRP-catalyzed dimerization of 2-methoxyphenol is about ten times faster with chlorine dioxide than with hydrogen peroxide. Two successive one-electron transfers from substrate AH (equations (2) and (3)) reduce compound I back to the native enzyme by way of the intermediate compound II. These enzyme intermediates are the same regardless of the oxidant used in equation (1).

A wide variety of compounds can serve as organic electron donors but readily-oxidized species such as phenols, amines and certain heterocyclic compounds such as ascorbic acid and indole are the
most common\textsuperscript{71}.

The catalytic cycle becomes more involved when higher concentrations of hydrogen peroxide are present, as the enzyme is oxidized further to compound III (oxyperoxidase, Scheme 11), which is unreactive with most electron donors except indoleacetic acid\textsuperscript{72}. Compound III can lose dioxygen to give the ferrous enzyme which is transformed to compound II by the reduction of one equivalent of hydrogen peroxide\textsuperscript{73}. It has also been found that HRP compound III can undergo a slow first-order decomposition to the native enzyme\textsuperscript{74}. Such catalatic activity has also been reported for lactoperoxidase\textsuperscript{75,76}, chloroperoxidase\textsuperscript{77} and bromoperoxidase\textsuperscript{78} and likely serves as a mechanism for the prevention of enzyme degradation by excess hydrogen peroxide. However, it is also accompanied by denaturation to a species formerly referred to as "compound IV". Studies with HRP have shown that the formation of compounds III and IV is dependent on the concentration of the peroxide rather than the ratio of peroxide to enzyme: compound III has been
quantitatively produced from lactoperoxidase with 0.5 mM hydrogen peroxide but 180 mM methyl hydrogen peroxide was required to completely denature HRP to compound IV.

The detection of a two-step conversion of the native enzyme to compound III by superoxide has led to the proposal that peroxidase may also be involved in the removal of this harmful species from the cell.

In order to examine if a peroxidase enzyme was capable of catalyzing the oxidative coupling of a dibenzylbutanolide to an aryltetralin (e.g. the conversion of yatein to deoxypodophyllotoxin), a preliminary investigation was conducted by Kutney et al. with HRP and the butanolide. This substrate, like all synthetic asymmetric compounds described in this thesis, was prepared as a racemate but is depicted as a single enantiomer for clarity. The substrate was chosen to be the readily available synthetic bis(hydroxybenzyl)butanolide rather than yatein because it was hoped that a lower oxidation potential resulting from the presence of phenolic hydroxyl groups would promote the formation of an aryltetralin product. Reaction of 58 with a solution of HRP and hydrogen peroxide resulted in the total consumption of the substrate and afforded the trans-1,2 aryltetralin 59 in 18% yield (Scheme 12). It was assumed that the poor yield was the result of excessively severe oxidizing conditions and could be improved by modifying the reaction parameters. Among the improvements considered was the substitution of HRP with peroxidases from the well-characterized and stable suspension culture of the "AC 3" line of C. roseus, which was available to us from a separate group in this laboratory. This culture had been found to contain several peroxidase isozymes which were involved in the formation of 3',4'-anhydrovinblastine (Scheme 7) and had properties similar to those of HRP.
1.5. BIOMIMETIC SYNTHESIS OF A 4'-DEMETHYLPODOPHYLLOTOXIN: PROPOSED WORK

Although the aryltetralin 59 was not intended to be a precursor to the podophyllotoxins owing primarily to the difficulties involved in selectively converting its ring B substituents to the required methylenedioxy group, we believed that the biotransformation of the butanolide 58 to 59 could serve as an excellent model system for future experiments in this area. Nevertheless, the experiment showed that the peroxidase-catalyzed cyclization of a dibenzylbutanolide offered access to the basic carbon skeleton of the podophyllotoxins. A route to these compounds and thence to etoposide involving organic synthesis and biotechnology thus seemed feasible. Since a promising result was obtained with a substrate already bearing a C(4") hydroxyl group, a 4'-demethylpodophyllotoxin such as 4 or 5, rather than podophyllotoxin itself, was selected as the immediate objective of this approach to etoposide.

In view of the low substrate specificity of peroxidase enzymes, it was hoped that the C. roseus peroxidase system, in the form of a CFE, would also catalyze the cyclization of 58 to 59 shown in Scheme 12. Our objective therefore was to first optimize the parameters for this model reaction and then to apply the results to similar substrates more suitable for the synthesis of the podophyllotoxins 4 or 5. In the event of the C. roseus CFE failing to provide the aryltetralin with the correct relative stereochemistry at C(1) and C(2), we intended to investigate biotransformations using a P. peltatum CFE. Since our line of P. peltatum culture had been found to produce podophyllotoxin 49, presumably

![Scheme 12](image-url)
by the biosynthetic route described in Scheme 8, an enzyme capable of making the appropriate C(6')-C(7") bond in the dibenzylbutanolide substrate may be present in this culture: finding the optimum substrate and conditions for this biotransformation (Scheme 13), however, would likely be a major challenge.

Should this approach prove unsuccessful in producing the podophyllotoxin stereochemistry at C(1), we intended to employ a synthetic pathway from the trans-1,2 aryltetralin (Scheme 14) to reach this goal. This route would involve the formation of a C(1)-C(2) double bond followed by hydrolysis of the lactone moiety, hydrogenation and lactonization. A very similar approach has been used in the final steps of a synthesis of (-)-deoxypodophyllotoxin (2), as shown in Scheme 15\textsuperscript{35}. It has been found that saponification of the lactone was a prerequisite for the addition of hydrogen to the appropriate face of the double bond in the subsequent step\textsuperscript{84}.

![Scheme 13](image.png)
Scheme 14.
Scheme 15.
2. **RESULTS AND DISCUSSION**

2.1. **SYNTHESIS OF Trans-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)BUTANOLIDE (58)**

Previous workers in this laboratory have already developed a synthetic route to the butanolide 58. The sequence described in this thesis is a modified version of their work, both in terms of reaction conditions and reagents.

The butanolide 58 was synthesized by a route involving a Stobbe condensation. Isovanillin (67) was treated with benzyl chloride and potassium carbonate (Scheme 16) to give the benzyl ether 68 in quantitative yield. The \(^1\)H-NMR spectrum of the product displayed the signals of the benzylic protons as a singlet at \(\delta 5.20\) and those on the phenyl ring as a multiplet at \(\delta 7.30-7.49\).

The benzyl ether 68 was then subjected to a Stobbe condensation with dimethylsuccinate to afford the conjugated hemisuccinate 69 in 73% yield. The carbonyl group was characterized by a

Scheme 16.
strong absorption at 1700 cm$^{-1}$ in the IR spectrum. The $^1$H-NMR spectrum showed the presence of the ester methyl singlet at $\delta 3.86$ and the olefinic proton signal at $\delta 7.81$. The spectrum indicated that only one geometrical isomer (probably cis) was present, as expected by comparison with the results reported for the analogous reaction of piperonal$^{89}$. The loss of material in this reaction was believed to be due to competing processes such as the Cannizzaro reaction and the reduction of dimethyl succinate by the alkoxide or enolate anions$^{63,90}$.

The double bond of the hemiester 69 was reduced with magnesium in methanol$^{91}$ to give the unconjugated hemisuccinate 70 in 89% yield. The $^1$H-NMR spectrum of the product showed H(2), H(3) and H(7') proton signals as either multiplets or doublets of doublets due to the creation of an asymmetric centre at C(2). The carbonyl band frequency in the IR spectrum, in comparison with that of 69, had increased to 1720 cm$^{-1}$, demonstrating the loss of conjugation of the ester group with the aromatic ring.

The reductive lactonization of the hemisuccinate 70 to the butanolide 71 (Scheme 17, Table 2) was initially performed using the potassium salt of 70 and calcium borohydride prepared in situ$^{92}$ (Method 1)$^{82}$. However, the crude reaction product was very impure and the yields were low. This step was improved (Method 2) by using the stronger reducing agent lithium borohydride$^{93,94}$ directly with the hemisuccinate 70. This procedure not only increased the yields of 71 but afforded a pure product following work-up. The butanolide 71 was characterized by its $^1$H-NMR spectrum which showed the two new methylene protons H(4) as doublets of doublets at $\delta 3.91$ and $\delta 4.21$, respectively. Formation of the lactone ring was demonstrated by the IR spectrum which showed the carbonyl band at a frequency of 1770 cm$^{-1}$, well above the value observed for the hemisuccinate 70.

![Scheme 17.](image)
### TABLE 2

**REDUCTIVE LACTONIZATION OF 70 TO 71**

<table>
<thead>
<tr>
<th>Method</th>
<th>Conditions</th>
<th>Yield 71 (%)</th>
</tr>
</thead>
</table>
| 1      | 1) KOH, H₂O; -H₂O  
        | 2) Ca(BH₄)₂, MeOH-EtOH, -20° to 25°, 4.5 d | 65 |
| 2      | 1) LiBH₄, THF, reflux, 2 h  
        | 2) HCl, 25°, 2.5 h | 88 |

Alkylation of the β-benzylbutanolide 71 with the bromide 72 (prepared by the literature procedure[^86]) gave the dibenzylbutanolide 73 in 80% yield (Scheme 18). The ¹H-NMR spectrum of the product, in comparison with that of 71, showed the loss of one H(2) signal but the expected *trans* orientation of the two substituents with respect to the lactone ring could not be confirmed owing to the close proximity of the complex H(2) and H(3) signals.

Finally, the benzyl protecting groups of the butanolide 73 were quantitatively cleaved by hydrogenolysis (Scheme 19), using palladium on carbon as a catalyst, to afford the bis(hydroxybenzyl)butanolide 58 in 87% yield. The ¹H-NMR spectrum of 58 (Appendix, Fig. 3) showed the C(3′) and C(4″) phenolic hydroxyl group singlets, which disappeared with D₂O addition, at δ5.42 and δ5.58 respectively. The individual signals were assigned by comparison with ¹H-NMR spectra of the 3′,4″-methylenedioxy and 3′,4″-dihydroxy analogues of 59[^82] and by the ¹H COSY NMR spectrum (Appendix, Fig. 4). The ¹³C-NMR spectrum showed two benzyl methylene carbon signals (C(7′)) and C(7″), which were identified by the attached proton test (APT). The El mass spectrum of 58 (Appendix, Fig. 5) clearly showed the molecular ion at m/z 388. The fragmentation pattern for the butanolide is shown in Fig. 6 of the Appendix.

In summary, the dibenzylbutanolide 58 was prepared from isovanillin in 6 steps in 40% overall yield.
Scheme 18.

1) LDA

71

2)

72

73

Scheme 18.
2.2. PRELIMINARY BIOTRANSFORMATIONS OF *TRANS*-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)BUTANOLIDE (58) AND *TRANS*-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3,4-METHYLENEDIOXYBENZYL)BUTANOLIDE (74)

Having a supply of the substrate dibenzylbutanolide 58, we attempted to cyclize it to the aryltetralin 59 as shown in Scheme 12 using hydrogen peroxide and CFE derived from our AC 3 line of *C. roseus*. Most of the parameters for the reaction (Table 3) were chosen on the basis of previous work with 3',4'-anhydrovinblastine\(^{42-45}\). For example, the culture had been found\(^\text{96}\) to have a high peroxidase activity and a very low rate of growth (as shown by its fresh weight, or the mass of filtered and drained cells) at an age of about 11 d, as shown on Graph 1. It is assumed that a culture with such properties would make the resultant CFE more reproducible between harvests. The peroxidase activity was found by measuring the amount of purpurogallin produced by the peroxidase-catalyzed oxidation of pyrogallol by hydrogen peroxide\(^\text{96}\).
Graph 1. Variation of peroxidase activity and fresh weight of AC 3 C. roseus culture with age.

TABLE 3
C. ROSEUS CFE BIOTRANSFORMATIONS OF BUTANOLIDES 58 AND 74

<table>
<thead>
<tr>
<th>Substrate</th>
<th>58</th>
<th>74</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH of Homogenization Buffer (pH$_h$)</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>pH of Biotransformation (pH$_r$)</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Hydrogen Peroxide (equivalents)</td>
<td>11</td>
<td>9.5</td>
</tr>
<tr>
<td>CFE (units peroxidase per mmol substrate)</td>
<td>140</td>
<td>245</td>
</tr>
<tr>
<td>Reaction Time (min)</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Compounds Isolated (%)</td>
<td>58 (10)</td>
<td>74 (25)</td>
</tr>
<tr>
<td></td>
<td>59 (58)</td>
<td>76(25)</td>
</tr>
</tbody>
</table>
Fig. 2. X-ray Diffraction Analysis of Aryltetralin Adduct 59-CHCl$_3$ (mirror image of structure shown in Scheme 12).

As noted in Table 3, the preliminary biotransformation of the butanolide 58 with C. roseus CFE afforded the aryltetralin 59 in 58% yield. The assigned structure of 59 was confirmed by X-ray diffraction analysis of a crystal of 59-CHCl₃, as shown in Fig. 1, where the mirror image of the structure shown in Scheme 12 is depicted. This does not contradict the previous structural assignment of 59 because even though the particular crystal of 59-CHCl₃ used for X-ray crystallography was optically active, circular dichroism analysis showed the bulk sample to be racemic. 

The ¹H-NMR spectrum of 59 (Appendix, Fig. 7) showed a broad doublet at δ4.07 (H(1)) and an aromatic proton signal pattern consisting exclusively of singlets, implying substitution at C(6') of the butanolide. The appearance of the H(2') proton signals as one singlet and the broad nature of the H(1) doublet indicated that ring E of 59 had essentially complete freedom of rotation. The individual proton assignments were consistent with the ¹H COSY NMR spectrum (Appendix, Fig. 8). The ¹³C-NMR APT spectrum of 59 showed signals corresponding to one benzyl methylene carbon (C(4)) and one benzyl methine carbon (C(1)). The El mass spectrum of 59 (Appendix, Fig. 9) showed a strong molecular ion peak at m/z 386 and a low degree of fragmentation, the mechanism for which is described in the Appendix (Fig. 1). Both of these features of the mass spectrum clearly distinguish the aryltetralin from its dibenzylbutanolide precursor.

The proposed mechanism for the cyclization of 58 to 59 is discussed in detail in Section 2.5.

The preliminary experiment thus showed that the bis(hydroxybenzyl)butanolide 58 could efficiently undergo the oxidative coupling reaction with C. roseus-produced enzymes and hydrogen peroxide to form the aryltetralin 59. If it is assumed that the carbon-carbon bond formation in converting 58 to 59 involves a radical coupling process, then both the C(3') and C(4") hydroxyl groups are desirable. However, it would be ideal, in terms of the podophyllotoxin-like products, if the substrate undergoing the carbon-carbon bond forming process already contains the 3',4'-methylenedioxy group. We therefore performed the CFE-catalyzed biotransformation of the yatein derivative 74 previously prepared in this laboratory by Kutney et al. The results of this study are summarized in Scheme 20 and Table 3.

The sole isolated product of the biotransformation, the conjugated butanolide 76, was characterized by its IR, ¹H-NMR and mass spectra. The IR spectrum showed a carbonyl band at 1745
cm\(^{-1}\), well below the value of 1780 cm\(^{-1}\) observed for 74 itself. This reduction in C-O stretching frequency can be explained by the conjugation of the electron-releasing ring E with the carbonyl group. The \(^1\)H-NMR spectrum of 76 (Appendix, Fig. 11) showed an olefinic proton signal at \(\delta 7.58\) and a hydroxyl singlet, well downfield at \(\delta 5.82\), which disappeared upon addition of D\(_2\)O. The double bond stereochemistry is believed to be \(E\), based on both the relative stabilities of Dreiding models of the \(E\) and \(Z\) isomers and the results of the oxidation of a similar yatein derivative\(^{97}\), but cannot be assigned conclusively using the available data. The individual proton assignments were supported by the effects

\[\text{Scheme 20.}\]
of decoupling H(7) and H(4). Since the aromatic region contained the signal pattern characteristic of a 1,3,4-tri-substituted ring (ring B) (Appendix, Fig. 3b), it was concluded that oxidative coupling had not occurred. Further evidence was provided by the EI mass spectrum of 76, which showed intense fragment ion peaks and a weak molecular ion peak at m/z 384 (Appendix, Fig. 12). By contrast, the EI mass spectra of aryltetralins (Figs. 9 and 23) exhibit minimal fragmentation. The major fragments at m/z 135 and 249 arise from the facile rearrangement shown in Fig. 13.

The conjugated butanolide 76 was probably formed by the isomerization of the hypothetical quinone methide intermediate 75 as shown in Scheme 20.

The overall recovery of organic products from the reaction mixture was low (50%). It was suspected that the material not accounted for (50%) had undergone oxidative demethylation to quinones, a known peroxidase-catalyzed process. The proposed mechanism for this oxidation is shown in Scheme 21. Following oxidation of the hydroxyl group, the radical 79 is attacked by a superoxide radical to form the peroxide anion 80. Successive protonations of 80 and the hydroperoxide 81 are followed by attack of the methyl group by water to eliminate hydrogen peroxide and produce methanol and the ortho-quinone 82. This hypothesis, which can clearly also be applied to biotransformations of 58, is supported by the fact that electron spin resonance spectroscopy has shown that superoxide as well as hydroxyl radicals are produced by lactoperoxidase in the presence of a high concentration of hydrogen peroxide. This process is accompanied by irreversible enzyme inactivation via oxidative cleavage of the porphyrin ring and release of the iron ion. The involvement of superoxide and hydroxyl radicals would thus also explain the incomplete conversion of the substrate in this biotransformation, where the initial hydrogen peroxide concentration (17 mM) was very high.

Although the biotransformation of 74 was not entirely conclusive on account of the poor recovery, the substrate did not appear to be a viable aryltetralin precursor under these conditions due to the absence of the desired aryltetralin product. Consequently, the biotransformation of the butanolide 74 by the C. roseus CFE was not studied further.
Scheme 21.

\[
\begin{align*}
\text{77} & \xrightarrow{-H^+} \text{78} \\
\text{80} & \xrightarrow{+\text{O}_2^-} \text{79} \\
\text{81} & \xrightarrow{+H^+} \text{82} \\
\end{align*}
\]
2.3. OPTIMIZATION OF CONDITIONS FOR BIOTRANSFORMATION OF TRANS-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)-BUTANOLIDE (58) TO 1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-6-HYDROXY-3-HYDROXYMETHYL-7-METHOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID γ LACTONE (59)

The results discussed in the previous section demonstrated that the C. roseus CFE would catalyze the oxidative coupling of the butanolide 58 to the aryltetralin 59 in good yield. Our next step therefore was the optimization of the biotransformation conditions, starting with an investigation of the effect of pH (the pH of the buffer used to prepare the CFE: Scheme 6) on the properties of the CFE. The ideal pH was determined as shown in Graph 2: a value of about pH 6.3 appeared to be a good compromise between high peroxidase activity and specific activity, or the quotient of peroxidase activity and soluble protein concentration. The protein concentration was determined spectrophotometrically by complexing the dissolved protein with a commercial dye solution.101,102

Graph 2. Effect of pH on peroxidase activity and specific activity.
The subsequent studies to establish the optimum parameters for the biotransformation of 58 were performed using the small-scale conditions described in Section 4.16. The stated yields of products and recovered starting material (collectively referred to as "Content (%)" in Graphs 3-6) represent calibrated HPLC values.

The ideal ratio of peroxidase to substrate was determined using an arbitrary amount of 2.0 molar equivalents of hydrogen peroxide and conducting the reaction at pH 6.3. As shown in Graph 3, 58 was very nearly consumed within 15 min when an amount of CFE corresponding to 400 units peroxidase per millimole of substrate was used. Increasing the peroxidase:substrate ratio or the reaction time (180 min, Graph 4) failed to completely convert 58 to 59; instead, the sole effect was to promote the reaction of aryltetralin 59 to 83, a species subsequently identified as the dimer of 59.

Graph 3. Effect of peroxidase:substrate ratio on biotransformation of 58. Reaction time = 15 min.
Graph 4. Effect of peroxidase:substrate ratio on biotransformation of 58. Reaction time = 180 min.

It was concluded that a peroxidase:substrate ratio of approximately 250 units per millimole of butanolide 58 would give optimum yields of the aryttetralin 59. Higher values promoted dimerization while lower values resulted in incomplete conversion of substrate.

A sample of the dimer 83 was obtained by the biotransformation of 58 and characterized by its $^1$H-NMR and mass spectra. The $^1$H-NMR spectrum of the dimer 83 strongly resembled that of the monomer 59 but there was a second signal of equal intensity for all protons shown by 59 except $H(5)$, which is not present. This doubling represents the proton signals of the two diastereomers of the dimer molecules since the monomer units are racemic. The dimeric nature of the compound was demonstrated more clearly by the mass spectrum obtained using desorption chemical ionization (DCI) with ammonia, which showed a peak at m/z 788 corresponding to (83 + NH$_4$)$^+$. There was a very weak molecular ion peak in the El mass spectrum (Appendix, Fig. 14) at m/z 770, as well as peaks at m/z 784, 798 and 812 resulting...
from the intermolecular transfer of methyl radicals. In addition, the high degree of fragmentation and the 
low intensity of the monomer peak at m/z 386, in relation to the fragments at m/z 167 and 154, were in 
sharp contrast to the pattern observed in the El spectrum of the monomer 59 (Appendix, Fig. 9). 
Unfortunately, neither the El nor the DCI spectra showed any significant fragments in the region 
spanned by the molecular weights of the monomer and the dimer, suggesting that the bond between 
the monomer units is very weak. The proposed mechanism for the dimerization of 59 to 83 is 
discussed in Section 2.5.

\[ \text{Chemical structure image of 83} \]

The minor compound 84 was also isolated in this biotransformation of 58. Its El mass spectrum was 
virtually identical to that of 59. The \(^1\)H-NMR spectrum was also quite similar except for the two ortho-
coupled (J = 8 Hz) proton signals at \(\delta 6.77\) and 6.85 which would suggest that the ring B hydroxyl group 
is at C(8) of the aryltetralin rather than at C(6) as in 59. Other significant features of the spectrum were 
the three separate methoxy proton signals at \(\delta 3.59, 3.80\) and 3.89 and the two meta-coupled (J = 2.5 
Hz) doublets at \(\delta 5.56\) and 6.09, which implied non-equivalence of the two sides of ring E due to the loss 
of its ability to rotate about the C(1)-C(1') bond. This was not surprising in view of the proximity of the 
C(8) hydroxyl to ring E. The low yield of this compound (0.53%) is readily explained by these major steric 
interactions of the C(8) hydroxyl with ring E.
The optimum hydrogen peroxide:substrate ratio was determined using 250 units peroxidase per millimole of 58 at pH 6.3. In the absence of hydrogen peroxide, no reaction occurred after 15 min (Graph 5) or 180 min (Graph 6). The yield of aryltetralins 59 and 83 then increased with hydrogen peroxide concentration but remained approximately constant at about 80% when more than 2.0 molar equivalents (3.1 mM) were present. However, these results clearly did not represent the exclusive requirements of the oxidative coupling reactions of 58 and 59 in view of the ability of the CFE alone to consume hydrogen peroxide. This property was demonstrated (Graph 7) when the CFE was stirred for 15 min with hydrogen peroxide prior to precursor addition. The yield of 59 was only 28% (vs 64% for the control, where precursor, hydrogen peroxide and CFE were added simultaneously) after 1 h but had increased sharply to 50% 1 h after a further 2.0 equivalents of hydrogen peroxide were added. These competing processes may have involved the catalytic decomposition of hydrogen peroxide (catalyzed by peroxidase, catalase or metal ions) or the oxidation of cell material.

It was concluded that 2.0 molar equivalents of hydrogen peroxide would give optimum yields of the aryletralin 59. Lower values resulted in incomplete biotransformation of 58 while higher ratios promoted the dimerization of 59 to 83. There appeared to be a distinct decrease in enzyme activity, as shown by the curves for 58 and 83 in Graphs 5 and 6, for ratios greater than 3 equivalents hydrogen peroxide: this was likely due to the oxidation of peroxidase compound II to catalytically-inactive forms of peroxidase such as compound III and possibly even the denatured enzyme (Scheme 11). Such processes would
Graph 5. Effect of hydrogen peroxide concentration on biotransformation of 58.

Reaction time = 15 min.
Graph 6. Effect of hydrogen peroxide concentration on biotransformation of 58.

Reaction time = 180 min.
Graph 7. Consumption of hydrogen peroxide by CFE reagent mixture.

not be unexpected considering the high concentrations of hydrogen peroxide employed in these experiments.

The effect of dilution of the biotransformation mixture was not investigated. It would be expected that upon dilution, the rate of dimerization of 59 would be reduced much more than the rate of formation of 59 in view of the difference in molecularity of the two reactions.

The optimum biotransformation pH (pH_r, Scheme 6) for the cyclization of 58 to 59 could now be reliably determined as the ideal peroxidase:substrate and peroxide:substrate ratios had been established. Up to this point, a value of pH 6.3 had been used and found to give good results but it was clearly desirable to identify the optimum value of pH_r more closely. This value (Graph 8) was found to be about 6.3 based on the yield of 59, using the optimum conditions established previously. The yield of 59 showed only minor variations over the range pH 6.0-6.6 but decreased markedly at more basic values.
Finally, it was determined that the CFE solution, when stored at 4°C, was quite stable with respect to both the oxidation of pyrogallol (peroxidase assay) and the biotransformation of 58, as shown in Graph 9. The age of the CFE was thus not a significant factor in the reproducibility of individual experiments. This was expected because peroxidases are very stable with respect to heat and storage time. In summary, it was found that the biotransformation of 58 to 59 could be accomplished most efficiently using the conditions described in Table 4.
Graph 9. Stability of *C. roseus* CFE. Reaction time = 180 min for biotransformation of 58.

**TABLE 4**

**OPTIMUM CONDITIONS FOR *C. ROSEUS* CFE-CATALYZED BIOTRANSFORMATION OF BUTANOLIDE 58 TO ARYL TETRALIN 59**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH&lt;sub&gt;H&lt;/sub&gt;</td>
<td>6.3</td>
</tr>
<tr>
<td>pH&lt;sub&gt;r&lt;/sub&gt;</td>
<td>6.3</td>
</tr>
<tr>
<td>Hydrogen Peroxide (equivalents)</td>
<td>2.0</td>
</tr>
<tr>
<td>CFE (units peroxidase per mmol 58)</td>
<td>250</td>
</tr>
<tr>
<td>Reaction Time (min)</td>
<td>180</td>
</tr>
</tbody>
</table>
2.4. LARGE SCALE BIOTRANSFORMATION OF \textit{TRANS}-2-(3,5-DIMETHOXY-4-
HYDROXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)BUTANOLIDE (58)

Having established a set of optimum parameters for the oxidative coupling of 58 to 59 using 15 mg of substrate, we wished to extend our findings to biotransformation conducted on a larger scale. As the properties of the \textit{C. roseus} CFE tend to vary between experiments, we employed slightly greater peroxidase:substrate and co-factor:substrate ratios in this biotransformation (Table 5) than those described in Table 4.

**TABLE 5**

**LARGE-SCALE BIOTRANSFORMATION OF DIBENZYL BUTANOLIDE 58**

<table>
<thead>
<tr>
<th>Substrate 58 (mg)</th>
<th>900</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH\textsubscript{i}, pH\textsubscript{r}</td>
<td>6.3</td>
</tr>
<tr>
<td>Hydrogen Peroxide (equivalents)</td>
<td>2.2</td>
</tr>
<tr>
<td>CFE (units peroxidase per mmol 58)</td>
<td>300</td>
</tr>
<tr>
<td>Reaction Time (min)</td>
<td>180</td>
</tr>
<tr>
<td>Compounds Isolated (%)</td>
<td>58 (6.2)</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>77.9</td>
</tr>
</tbody>
</table>

Although the yield of 59 was similar to that expected from the small-scale optimization studies, no dimeric aryltetralin 83 was isolated and over 20% of the starting material could not be accounted for. This difference may have been the result of an oxidation of starting material or products to quinones (Scheme 19). A control experiment conducted without hydrogen peroxide verified the efficiency of the standard extraction procedure as a 97% recovery of 58 was obtained.
The interesting minor product 85 isolated in this experiment was characterized by IR, $^1$H-NMR and mass spectroscopy. Its IR spectrum showed, in addition to a hydroxyl O-H stretching frequency at 3539 cm$^{-1}$ and the lactone carbonyl C-O stretch at 1774 cm$^{-1}$, a second carbonyl band at 1673 cm$^{-1}$ which was associated with the C(4') dienone. The El mass spectrum (Appendix, Fig. 16) showed the molecular ion as the base peak at m/z 386 and virtually no fragmentation. The $^1$H-NMR spectrum (Appendix, Fig. 15) showed three distinct methoxy proton signals, two H(6) proton signals (assigned by decoupling H(7)) and two sharp doublets (J = 2.8 Hz) at $\delta$6.13 and 6.25 which represent H(2') and H(6'). The chemical shift and coupling constant values of H(2') and H(6') were very similar to those reported for the related compound 86$^{104}$.

![Structural formulae of 85 and 86](image)

2.5. PROPOSED MECHANISMS OF BIOTRANSFORMATION OF TRANS-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)-BUTANOLIDE (58) AND 1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-6-HYDROXY-3-HYDROXYMETHYL-7-METHOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID $\gamma$ LACTONE (59)

The cyclization of the dibenzylbutanolide 58 is believed to proceed through a quinone methide intermediate: the following mechanism, based on the oxidation of related compounds with HRP$^{105}$, is...
Following oxidation of the peroxidase by hydrogen peroxide (p. 23, equations (1)-(4)), the C(4") hydroxyl of 58 is oxidized to the corresponding phenoxyl radical 87. Oxidation of the C(4'), rather than the C(3'), hydroxyl would be expected first owing to the ortho,para-tri-substitution of ring E which should make this radical more stable than the one derived from ring B. Hydrogen atom H(7") is then removed to form the quinone methide 88. Studies of the HRP-catalyzed oxidation of etoposide\textsuperscript{106} have demonstrated that quinone methide formation was the result of disproportionation of phenoxyl radicals, rather than hydrogen atom abstraction by peroxidase: a similar mechanism is postulated here.

The resultant intermediate 88 subsequently loses the C(3') hydroxyl hydrogen atom to generate the ring B radical 89 which cyclizes to the dienone radical 90. Alternately, the quinone methide 88 may proceed directly to the final product 59, without any further oxidation, through an intramolecular nucleophilic addition process. In either case, these last two steps are probably very rapid since isomerization of the quinone methide 88 to the conjugated lactone was not observed in any experiment. Finally, both ring systems are transformed back to the corresponding phenols. The ring B dienone likely undergoes a tautomerization while the ring E radical gains a hydrogen atom. The latter process may involve the oxidation of either hydrogen peroxide\textsuperscript{76} or a phenolic hydroxyl.

The formation of the dimer 83 is believed to occur, as shown in Scheme 23, by the coupling of two ring B phenoxyl radicals 92 generated by the oxidation of the C(3') hydroxyl by peroxidase. No reaction was observed when 59 was stirred with hydrogen peroxide in the absence of CFE.

The proposed mechanism for the formation of the spirodienone 85, as shown in Scheme 24, is based on the very similar oxidative coupling step postulated in the biosynthesis of various morphine alkaloids\textsuperscript{107,108}. 

proposed (Scheme 22).
Scheme 22.
Scheme 23.

\[ \text{Scheme 23.} \]
Scheme 24.

58

H$_2$O$_2$

Peroxidase

93

85
2.6. **TRANS-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3,4-DIHYDROXY-\(\alpha\)-HYDROXYBENZYL)BUTANOLIDE (94)**

### 2.6.1. SYNTHESIS

The results of the biotransformations of the butanolides 58 and 74 showed that a 3',4'-methylenedioxy group, present in all podophyllotoxins, would not permit the formation of the corresponding aryltetralin. A 4'-demethyl derivative of 58 thus seemed to be worthy of further study because a hydroxyl was apparently required at C(3') and a methylenedioxy group is most readily prepared from a catechol. In addition, we wished to examine the influence of a butanolide C(7') hydroxyl group on the stereochemistry of the cyclized product. We felt that such a substrate (94) could be prepared most readily by a route involving the tandem conjugate addition method of Ziegler and Schwartz\(^{109,110}\) rather than by a modification of the synthetic pathway employed for 58. The planned route is shown in retrosynthetic form in Scheme 25. The key step is the one-pot conjugate addition of the diphenylthioacetal 97 to but-2-en-4-olide (98) followed by alkylation with the previously-prepared bromide 72. The conversion of the resulting dibenzylbutanolide 96 to the catechol 94 should then be a straightforward matter.

The dithioacetal 97 was obtained from piperonal (32) as shown in Scheme 26 in 98% yield\(^84\). The crude product was pure according to its \(^1\)H-NMR spectrum, which showed a ten-proton multiplet at \(\delta7.23-7.42\) (phenyl protons) and a new signal at \(\delta5.37\) corresponding to the benzyl proton. The mass spectrum showed a very weak molecular ion peak at m/z 352 but a very strong signal at m/z 243 (M+\(-\text{SPh}\)).

The dithioketal 96 was then prepared in 55% yield as shown (Scheme 27) using but-2-en-4-olide obtained by a standard procedure\(^111\). The \(^1\)H-NMR (Appendix, Fig. 17) and \(^1\)H COSY NMR (Appendix, Fig. 18) spectra of 96 were consistent with the assigned structure. The IR spectrum showed a strong carbonyl band at 1770 cm\(^{-1}\), a frequency substantially higher than that observed for the butenolide (1740 cm\(^{-1}\)). The El mass spectrum (Appendix, Fig. 19) showed no significant identified peaks with m/z values greater than 584 ((M+\(-\text{SPh}\)+H) but the fast atom bombardment ionization (FAB) mass spectrum revealed a weak molecular ion at m/z 693.
Scheme 25.

Scheme 26.
Considerable problems were encountered in the subsequent oxidation of the dithioketal 96 to the ketone 100 (Scheme 28, Table 6). Treatment of dithioketal 96 with perchloric acid (Method 1)\textsuperscript{112} gave complex polar mixtures and poor yields of 100 along with the hydroxy ketone 101 as a minor product. Varying the reaction time and the amount of perchloric acid failed to improve these results. Oxidation of 96 with sulfuryl chloride and wet silica gel (Method 2)\textsuperscript{113} gave complex mixtures containing no readily identifiable products. Oxidation with iodine in methanol followed by acid hydrolysis (Method 3)\textsuperscript{114}, however, gave good yields of the ketone 100, which was characterized by \textsuperscript{1}H-NMR, mass and IR spectroscopy. The \textsuperscript{1}H-NMR spectrum of 100, in comparison with that of the dithioketal 96, showed a reduction in the number of phenyl protons to five and a considerable downfield shift of the H(3) signal to 34.01. The El mass spectrum exhibited a molecular ion peak at m/z 490. The hydroxy ketone 101 was
also characterized by its $^1$H-NMR spectrum, which showed a phenolic hydroxyl singlet at $\delta 5.37$ (disappeared with D$_2$O) and no phenyl proton signals. Its EI mass spectrum showed a molecular ion peak at m/z 400. The IR spectra of both ketones 100 and 101 showed a second carbonyl band at 1670 cm$^{-1}$.

Scheme 28.
TABLE 6
HYDROLYSIS OF DITHIOKETAL 96

<table>
<thead>
<tr>
<th>Method</th>
<th>Conditions</th>
<th>Yield 100 (%)</th>
<th>Yield 101 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HClO₄, EtOAc</td>
<td>44</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>SO₂Cl₂, silica gel, H₂O</td>
<td>no products isolated</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1) I₂, MeOH, reflux</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2) aq. HCl-THF</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The ketone 100 was then subjected to hydrogenolysis (Scheme 29, Table 7) to afford the hydroxy ketone 101 (46%) and the benzyl alcohol 102 (30%). Although a large excess of catalyst was used in comparison with the de-benzylation of 73 (Scheme 19), a considerable amount of starting material (15%) was recovered from the reaction mixture. It is believed that catalyst poisoning caused by residual sulfur compounds in the starting material had produced the incomplete conversion.115,116

The ¹H-NMR spectrum of the alcohol 102 showed it to be a 3.3:1 mixture of alcohols epimeric at C(7'). The major isomer was tentatively identified as the alcohol with a β orientation of the C(7') hydroxyl for the depicted enantiomer, based on the relative accessibility of the faces of the C(7') carbonyl group in the most favourable conformation of 100, according to Dreiding models, and the results of reductions of closely-related dibenzylbutanolides.¹¹² The ¹H-NMR spectrum of 102 showed doublets at δ1.94 and 2.00 and a phenolic hydroxyl singlet at δ5.40, all of which disappeared upon D₂O addition. The doublets correspond to the C(7') α and β hydroxyl protons, respectively. The H(3)-H(7') coupling constants of the two epimers (7.6 and 6.7 Hz for minor and major isomers, respectively) were too similar to be useful in assigning the C(7') stereochemistry of the individual isomers. This uncertainty does not present a problem because both C(4) epimers of 4'-demethylpodophyllotoxin serve equally well for the synthesis of etoposide (Scheme 5).
Nevertheless, we did not wish to continue the synthesis of the catechol 94 using epimeric mixtures. We thus sought to obtain a single isomer of 102 by treating the ketone 100 with sodium borohydride (Scheme 33, Table 7). This reaction afforded the alcohol 102 in 96% yield: its $^1$H-NMR spectrum indicated that it was present as a 6:1 mixture of the same epimers. Using this mixture, we treated 102 with boron trichloride according to a published procedure in order to cleave the methylenedioxy group, as shown in Scheme 30. The alcohol 102 was found to react quantitatively to afford the catechol 94 (24%) and the aryltetralin 103 (40%) as the sole isolated products. Both compounds were obtained as single isomers based on their $^1$H-NMR spectra.
Scheme 30.

The catechol 94 was characterized by NMR and mass spectroscopy. Its $^1$H-NMR spectrum (Appendix, Fig. 20) showed a broad two-proton signal at $\delta 7.80$ (catechol hydroxyl protons), a singlet at $\delta 6.91$ (C(4*) hydroxyl) and a doublet at $\delta 4.55$ (C(7*) hydroxyl), all of which disappeared upon D$_2$O addition. The EI mass spectrum (Appendix, Fig. 21) shows a weak molecular ion at m/z 390 and several strong fragments.

The aryltetralin 103 was identified by its $^1$H-NMR and mass spectra. The carbon skeleton of such compounds is commonly referred to as being of the "retro-lignan" type. The $^1$H-NMR spectrum of 103
(Appendix, Fig. 22) showed singlets at $\delta 3.18$ and 3.87 (methoxy protons), singlets at $\delta 7.12$, 7.54 and 7.56 (hydroxyl protons) which disappeared upon D$_2$O addition and a total of only four aromatic protons. The tri-substituted ring B aromatic signal pattern (Appendix, Fig. 3) was present but the H(2") signal appeared as only a single proton singlet at $\delta 6.65$. The EI mass spectrum of 103 (Appendix, Fig. 23), in contrast to that of the catechol 94, showed a very strong molecular ion peak at m/z 372 and a low degree of fragmentation.

The reaction was repeated at -100° C using less boron trichloride and a shorter reaction time (Scheme 31) in order to determine if the by-product 103 was formed from the catechol 94 or independently from the starting material 102. The only product observed by TLC and subsequently isolated was the aryltetralin 104 (6% yield), indicating that the cyclization proceeded directly from the alcohol 102. Unreacted alcohol 102 (69%) was also isolated. The purpose of the milder hydrolysis procedure was to determine if the poor recovery in the initial experiment was due to product decomposition during work-up. Since no catechol was produced according to TLC and the recovery (75%) was not greatly improved, it was concluded that the cause of the problem was not the work-up but other reactions involving boron trichloride.

Scheme 31.
Scheme 32.
The aryltetralin 104 was characterized by its $^1$H-NMR and mass spectra. The $^1$H-NMR spectrum of 104 showed three distinct methoxy proton signals and a singlet at $\delta 5.93$ corresponding to the methylenedioxy group. The El mass spectrum showed an intense molecular ion peak at m/z 384 and minimal fragmentation.

The mechanism of the formation of 104, based on these findings, is proposed to involve an intramolecular Friedel-Crafts alkylation$^{118}$ as shown in Scheme 32. The presence of a benzyl cation (106) as an intermediate would explain why only one stereoisomer of the product was obtained from a starting material consisting of a mixture of epimers. The pathway leading to 103 is identical except that the intermediate 108 undergoes further reaction with boron trichloride to afford the catechol.

Although the catechol 94 could thus be prepared from the ketone 100, the synthetic route used to arrive at this compound was unsatisfactory on account of the poor yield and conversion of the hydrogenolysis step. We reasoned that since boron trichloride should be able to cleave the C(4") benzyl ether$^{119}$ we could omit the hydrogenolysis reaction altogether and carry out the cleavage of the methylenedioxy and benzyl ether groups in one step using the alcohol 109, which should be easily prepared from the ketone 100 (Scheme 33).

Reduction of the ketone 100 with sodium borohydride gave a 4.9:1 epimeric mixture of the alcohol 109 (Table 7, Entry 3) in quantitative yield. The $^1$H-NMR spectrum of 109 demonstrated the presence of a benzyl group (five phenyl protons at $\delta 7.24-7.51$ and two benzylic protons at $\delta 5.00$) and a benzylic hydroxyl group (doublet at $\delta 1.96$ which disappeared upon D$_2$O addition). It was assumed that the C(7') $\beta$ hydroxyl epimer was again the major isomer: in fact, a subsequent reaction of 109 with boron trichloride produced 102 as an approximately 5:1 mixture of the same isomers detected previously.

We attempted to improve the ratio of the epimers of 109 by reducing the ketone 100 with the more selective reagent lithium aluminum tri-$t$-butoxy hydride (Entry 4)$^{36}$. This reaction afforded a slightly better proportion of the major isomer of 109 but the overall recovery (86%) of starting material and product was significantly lower even though the conversion of ketone 100 was only 70%. Consequently, the sodium borohydride procedure was adopted as the best method for the reduction of ketone 100 to the alcohol 109.
TABLE 7  
REDUCTION OF KETONES 100 AND 101

<table>
<thead>
<tr>
<th>Entry</th>
<th>Ketone</th>
<th>Conditions</th>
<th>Alcohol</th>
<th>OH(7')</th>
<th>Yield Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>101</td>
<td>NaBH₄, 0°C</td>
<td>102</td>
<td>6:1</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>H₂/Pd-C</td>
<td>102</td>
<td>3.3:1</td>
<td>30*</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>NaBH₄, 0°C</td>
<td>109</td>
<td>4.9:1</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>LiAI(t-BuO)₃H,</td>
<td>109</td>
<td>5.7:1</td>
<td>59*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-78°C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*) Incomplete reaction
The alcohol 109 was then treated with boron trichloride (Scheme 34) as described in Table 8 but the yield of the catechol 94 remained virtually unchanged regardless of the reaction time or the amount of boron trichloride used. However, a neutral chromatographic column packing (Method 4) was found to noticeably improve the recovery, suggesting that some decomposition may have occurred on the silica.

![Scheme 34](image)

**TABLE 8**

**CONVERSION OF ALCOHOL 109 TO CATECHOL 94 WITH BORON TRICHLORIDE**

<table>
<thead>
<tr>
<th>Method</th>
<th>Boron trichloride (equivalents)</th>
<th>Time (min)</th>
<th>Yield 94 (%)</th>
<th>Column packing</th>
<th>Column recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>120</td>
<td>26</td>
<td>Silica</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>59</td>
<td>22</td>
<td>Silica</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>30</td>
<td>19</td>
<td>Silica</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>40</td>
<td>27</td>
<td>Florisil</td>
<td>67</td>
</tr>
</tbody>
</table>
columns. It is suspected that the material not accounted for represents products which were demethylated by boron trichloride\textsuperscript{119} and remained adsorbed to the column packing on account of their high polarity.

In summary, the synthesis of the catechol \textit{94} was accomplished in 5 steps in 12\% overall yield from piperonal. The low yield was the result of problems encountered in the removal of the methylenedioxy group.

\subsection*{2.6.2. BIOTRANSFORMATION}

We then proceeded to investigate the biotransformation of the catechol \textit{94} by the \textit{C. roseus} CFE with the intention of performing the oxidative coupling of \textit{94} to the aryltetralin \textit{110} (Scheme 35). Since catechols are known to react very readily with peroxidases to form ortho-quinones\textsuperscript{120} and the corresponding diacids\textsuperscript{98} (Scheme 36), the biotransformation of \textit{94}, as described in Table 9, was conducted under oxidizing conditions considerably milder than those employed for \textit{58}. In addition to lower peroxidase:substrate ratio, a higher pH was employed in order to achieve a lower peroxidase reactivity (Graph 8). Nevertheless, the color of the reaction mixture changed from an initial pale yellow to dark purple within one minute, suggesting extensive oxidation of starting material to quinones. No such color change had been observed in the biotransformation of \textit{58}.A control experiment (Scheme 37) was also performed in order to determine if hydrogen peroxide, in the absence of CFE, was able to oxidize the catechol \textit{94} under the conditions used in the biotransformation. Extraction of the reaction mixture gave 96\% recovery of a material consisting exclusively of \textit{94} according to TLC analysis and \textsuperscript{1}H-NMR spectroscopy. No color change was observed. It was concluded that hydrogen peroxide alone was unable to oxidize the catechol under the given conditions.

Isolation of the biotransformation products was carried out as shown in Scheme 37. Analysis of the ethyl acetate extract by TLC showed the presence of only \textit{94} and very polar unidentified compounds. Addition of sodium borohydride (Table 10, Entry 1) gave a second major component according to TLC even prior to the reaction being quenched with dilute hydrochloric acid. The \textsuperscript{1}H-NMR spectrum and
Scheme 35.

Scheme 36.
TABLE 9

C. ROSEUS CFE BIOTRANSFORMATION OF CATECHOL 94

<table>
<thead>
<tr>
<th>Substrate 94 (mg)</th>
<th>73.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH&lt;sub&gt;H&lt;/sub&gt;, pH&lt;sub&gt;R&lt;/sub&gt;</td>
<td>7.0</td>
</tr>
<tr>
<td>Hydrogen peroxide (equivalents)</td>
<td>2.0</td>
</tr>
<tr>
<td>CFE (units peroxidase per mmol 94)</td>
<td>120</td>
</tr>
<tr>
<td>Reaction Time (min)</td>
<td>10</td>
</tr>
<tr>
<td>Ethyl Acetate Extract (mg)</td>
<td>67.9</td>
</tr>
<tr>
<td>Methanol Extract (mg)</td>
<td>205.1</td>
</tr>
</tbody>
</table>

TLC properties of this compound were identical with those of the aryltetralin 103. The fact that 103 was obtained under basic reducing conditions suggests that it was already present in the ethyl acetate extract, probably as an ortho-quinone, rather being formed directly from 94 by the hydrochloric acid. However, the yield of 14% may represent partial cyclization of 94 to 103 by this latter route. Its absence in the control experiment also indicates that its formation was enzyme-dependent. The proposed mechanism for this process is shown in Scheme 38 and is essentially identical to the one shown in Scheme 32. Since the biotransformation was conducted at a neutral pH, the reaction is probably promoted by the strong electron-withdrawing nature of the hypothetical quinone group.

Only the substrate 94 and partially methylated 94 (114) were obtained when the ethyl acetate extract was treated with diazomethane (Entry 2). The butanolide 114 was isolated as a single spot by TLC analysis and characterized by its IR, <sup>1</sup>H-NMR and mass spectra. The IR spectrum showed hydroxyl bands at 3600 and 3530 cm<sup>-1</sup> corresponding to the benzylic and aromatic hydroxyl groups, respectively. A carbonyl band at 1770 cm<sup>-1</sup> was also present. The <sup>1</sup>H-NMR spectrum was nearly identical to that of 94 except for two additional methoxy proton signals and an apparent doubling of all signals which suggested two very similar compounds. The presence of 114 as a mixture of two isomers
was supported by its El mass spectrum which showed the molecular ion of 114 as the base peak at m/z 418.

Successive treatments of the ethyl acetate extract with large excesses of sodium borohydride and freshly-prepared diazomethane (Entry 3) afforded 115 and 116 as the sole identified products. The butanolide 115 was characterized by a 1H-NMR spectrum which showed a broad doublet at 24.22 (H(4)) and an aromatic signal pattern consistent with the tri-substituted aromatic ring at C(4). The El mass spectrum of 113 clearly showed the molecular ion at m/z 414. The 1H-NMR and mass spectral data for 116 did not permit an unambiguous structural assignment to be made. The relative yields of 115 and 116 strongly suggested that the compound was an artifact of the reduction and methylation processes.
TABLE 10

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reagent(s)</th>
<th>Products</th>
<th>Yield (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sodium Borohydride</td>
<td>94</td>
<td>12</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>103</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Diazomethane</td>
<td>94</td>
<td>9</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>114</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1) Sodium Borohydride</td>
<td>115</td>
<td>20</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>2) Diazomethane</td>
<td>116</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

*) Total recovery of material from chromatographic column or preparative TLC plate.

and actually represented the catechol 94. It is proposed that the large excess of sodium borohydride in an aqueous medium resulted in the hydrolysis of the lactone to the hydroxy acid. Such a structure would be consistent with the polarity of this compound as neat acetone was required to elute 116 from the chromatographic column of silica. Further support is provided by the presence of a broad O-H stretch at 3320 cm\(^{-1}\) and a carbonyl band at 1670 cm\(^{-1}\) in the IR spectrum, both of which resemble the characteristic stretching frequencies of a carboxylic acid. Both the El and DCI mass spectra of 116 exhibited a very high degree of fragmentation and peaks of low intensity, as expected for an acyclic compound. The main objection to a hydroxy acid structure is that the acid moiety produced by saponification should have reacted with diazomethane in the subsequent step.
Scheme 38.

\[ 94 \xrightarrow{\text{H}_2\text{O}_2, \text{CFE}} 117 \]

\[ 119 \xrightarrow{1) - \text{H}_2\text{O}} 118 \xrightarrow{2) - \text{H}^+} \]

\[ \text{NaBH}_4, \text{H}_2\text{O} \]

103
The nature of the unrecovered biotransformation products of 94 is not known. The total recovery of material from the chromatographic separation of the reduced and methylated ethyl acetate extract (Entry 3) was 82% but only 30% of this material showed aromatic proton signals by \textsuperscript{1}H-NMR spectroscopy. Consequently, the methanol extract of the biotransformation mixture was expected to contain a significant portion of the biotransformation products but TLC analysis showed only baseline (Rf = 0) material after treatment with sodium borohydride or diazomethane. Treatment with excess sodium borohydride followed by methylation and isolation of material with an Rf value greater than zero by silica
TLC analysis gave only minor amounts of an unidentified compound, assumed to be derived from the CFE. It is speculated that oxidative cleavage of ring B to the diacid (Scheme 36) and dimerization processes involving ring B may have been involved.

Although the experiment was not entirely conclusive on account of the poor recovery of starting material and products, it gave no evidence of the formation of either the aryltetralin 110 or its ortho-quinone 120 (Scheme 39). Even if the cyclization of 94 to 110 had occurred but had been followed by further oxidation to 120, the aryltetralin 110 or a methylated derivative should have been isolated in one of the experiments described in Scheme 36. It was thus concluded that the oxidation of ring B to some unidentified species was too rapid to permit the formation of the quinone methide intermediates required for cyclization to 110. The low recovery values were probably also the result of a highly reactive substrate resulting from the presence of a catechol function. Therefore, the catechol 94 does not appear to be a suitable starting material for the preparation of lignan-type aryltetralins by C. roseus CFE-catalyzed oxidation.

Scheme 39.
3. CONCLUSION

Although this thesis did not realize the goal of a new synthetic route to etoposide, it does shed some light on both the preparation of various butanolide intermediates and their oxidative coupling, as catalyzed by the *C. roseus* CFE. Furthermore, it clearly demonstrates the potential of plant cell cultures in organic synthesis by the consistently good yields obtained in the biotransformation of the butanolide 58 to the aryltetralin 59.

The results obtained from the biotransformations of the butanolides 58, 74 and 94 suggested that the following properties were required by such substrates in order to be efficiently converted to a lignan-type aryltetralin. First, the apparent failure of 74 to form an aryltetralin demonstrated that a hydroxyl must be present at C(3') on ring B. Presumably, the oxidation of this group by peroxidase leads to the formation of a phenoxy radical which enhances the reactivity of ring B and thus makes the cyclization process competitive with the isomerization of the hypothetical intermediate quinone methide to an conjugated lactone such as 76. However, ring B cannot have hydroxyls at both C(3') and C(4') as the oxidation of such a substrate to unidentified compounds is apparently much faster than the rate of formation of the C(6')-C(7'') bond. Finally, it was found that a C(7') hydroxyl was undesirable as it promoted an intramolecular Friedel-Crafts alkylation leading to a retro-lignan structure.

The results of this work thus suggest that the ideal substrate for the synthesis of a lignan-type aryltetralin carbon skeleton by oxidative coupling with *C. roseus* CFE would be an analogue of 94 in which the C(4') substituent is a water-stable protecting group which could be cleaved following biotransformation. The synthesis and biotransformation of such a compound are currently under investigation. In addition, the question of how to obtain the podophyllotoxin cis-1,2 stereochemistry in the aryltetralin product still remains to be solved.
4. EXPERIMENTAL

4.1. GENERAL

Melting points were determined using a Reichert melting point apparatus and are uncorrected. The recrystallization solvents are given in brackets. Infrared spectra were recorded on Perkin Elmer 1710, 710 or 710B spectrometers, using sodium chloride cells, as a chloroform solution (0.1 mm path length), thin film or Nujol mull. Band frequencies are reported relative to polystyrene (1601 cm\(^{-1}\)). Ultraviolet spectra were recorded as methanol solutions on a Cary 15 spectrometer using 1 cm quartz cells. \(^1\)H-NMR spectra were recorded on Bruker WH-400, Bruker AC 200 or Varian XL-300 spectrometers. Chemical shift values are reported in ppm relative to tetramethylsilane as an internal standard. \(^1\)H COSY NMR spectra were recorded on a Bruker AC 200 spectrometer. \(^13\)C-NMR spectra were recorded on a Varian XL-300 spectrometer at 75.3 MHz or a a Bruker AC-200 spectrometer at 50.2 MHz. Signals with a negative intensity in the attached proton test (APT) are indicated as such by "(-)" and imply the attachment of an odd number of protons. Mass spectra were recorded on AEI-MS-9 (low resolution) or KRATOS-MS-50 (high resolution) spectrometers, employing electron impact, fast atom bombardment or desorption chemical ionization methods. Elemental analyses were performed using combustion analysis by Mr. P. Borda, Microanalytical Laboratory, University of British Columbia. Determination of structures by X-ray crystallography was performed by the X-ray Crystallography Laboratory, University of British Columbia.

Cell suspension culture of the AC 3 line of \emph{C. roseus} was obtained from Biological Services of the Department of Chemistry, University of British Columbia. Column chromatography (referred to as "flash chromatography")\(^{121}\) was performed using columns of silica gel (230-400 mesh, Merck art. 9385) or Florisil (60-100 mesh, Fisher F-100) with air or nitrogen gas pressure to obtain a rapid flow rate. Thin-layer chromatography was performed using commercial aluminum-backed silica gel plates (Merck art. 5554). Visualization was accomplished by spraying with 5% ammonium molybdate in 10% aqueous sulfuric acid followed by heating. High-pressure liquid chromatography was performed using a Waters C-18 "Radial Pak" liquid chromatography cartridge, a Waters 440 Absorbance Detector set at 280 nm and a methanol/water eluent. Circular dichroism analysis was performed using a JASCO J-20 Automatic
Anhydrous ether and tetrahydrofuran were prepared by distillation from sodium benzophenone ketyl. Anhydrous dichloromethane and diisopropylamine were prepared by distillation from calcium hydride. Anhydrous methanol was prepared by distillation from magnesium. Boron trichloride was used as a dichloromethane solution prepared from the condensed gas. Diazomethane was prepared from N-methyl-N-nitrosotoluene-\(p\)-sulfonamide by the published procedure\textsuperscript{122}. Aqueous hydrogen peroxide was standardized by iodometric titration with sodium thiosulfate\textsuperscript{123}.

4.2. 3-BENZYL OXY-4-METHOXYBENZALDEHYDE (68)

\[
\text{PhCH}_2\text{O} \quad \text{CHO} \\
\text{CH}_3\text{O} \\
\text{5} \quad \text{6}
\]

A suspension of isovanillin (67, 24.9 g, 164 mmol), potassium carbonate (24.8 g, 179 mmol), benzyl chloride (24.8 g, 194 mmol), sodium iodide (1.0 g, 6.7 mmol) and ethanol (75 mL) was refluxed for 4.5 h while stirring with a mechanical stirrer. Water (50 mL) was added and the ethanol removed \textit{in vacuo}. The slurry was poured into a mixture of 1 M sodium hydroxide (125 mL) and ice (50 g). The solids were filtered off and washed with ice-cold water (3 x 25 mL). Drying \textit{in vacuo} gave the benzyl ether 68 (40.2 g, 100%) as a tan solid. A sample was recrystallized from ethyl acetate/hexanes for analysis.

Physical properties of 68

Mp 60.0-60.5°C (ethyl acetate/hexanes)

\text{IR } \nu_{\text{max}} (\text{CHCl}_3): 1690 \text{ cm}^{-1}.

\text{UV } \lambda_{\text{max}} (\log \varepsilon): 273 (4.03), 305 (3.91) \text{ nm}.

\text{H-NMR (300 MHz, CDCl}_3): 3.97 (3 H, s, -OCH}_3), 5.20 (2 H, s, -OCH}_2\text{Ph}), 7.01 (1 H, d, H(5), J = 8 \text{ Hz}).
7.30-7.49 (7 H, m, aromatic), 9.81 (1 H, br s, -CHO).

EI MS m/z: 242 (M+), 214, 181, 168. Exact mass calculated for C\(_{15}\)H\(_{14}\)O\(_3\) 242.0943; found: 242.0942.

Elemental analysis: calculated for C\(_{15}\)H\(_{14}\)O\(_3\): C (74.36), H (5.82); found: C (74.25), H (5.82).

4.3. **E-2-(3-BENZYLOXY-4-METHOXYBENZYLIDENE)BUTANEDIOIC ACID 1-METHYL ESTER** (69)

\[ \text{PhCH}_2\text{O} \quad \text{2'} \quad \text{7'} \quad \text{CO}_2\text{CH}_3 \\
\text{CH}_3\text{O} \quad \text{6'} \quad \text{3} \quad \text{CO}_2\text{H} \]

A solution of sodium methoxide was prepared by the careful addition of sodium (14.6 g, 633 mmol) to dry methanol (250 mL) under nitrogen. A solution of the benzyl ether 68 (95.7 g, 395 mmol) in dimethylsuccinate (81.6 g, 558 mmol) was added dropwise over 40 min at reflux. After an additional 70 min of stirring at reflux, the bulk of the solvent was removed in vacuo. The suspension was cooled to 0°C and acidified with 6 M hydrochloric acid. The solids were removed by filtration and the filtrate extracted with dichloromethane (2 x 400 mL). The solids were added to the organic extract, washed with brine (400 mL), dried over magnesium sulfate, filtered and evaporated in vacuo to yield an oily yellow solid which was recrystallized from ethyl acetate/hexanes to give the hemisuccinate 69 (102.1 g, 73%) as a yellowish powder.

Physical properties of 69

Mp 123-125° C (ethyl acetate/hexanes)

IR \( \nu_{\text{max}} \) (CHCl\(_3\)): 1700 cm\(^{-1}\).

UV \( \lambda_{\text{max}} \) (log \( \epsilon \)): 290 (4.03), 307 (4.03) nm.

\(^1\)H-NMR (400 MHz, CDCl\(_3\) \( \delta \)): 3.51 (2 H, s, H(2)), 3.86, 3.93 (3 H each, s, s, -CO\(_2\)CH\(_3\), Ar-OCH\(_3\)), 5.22 (2
H, s, -OC\textsubscript{6}H\textsubscript{5}Ph), 6.94 (1 H, d, H(5'), J = 8 Hz), 7.05 (1 H, dd, H(6'), J = 8, 1 Hz), 7.10 (1 H, d, H(2'), J = 1 Hz), 7.37-7.45 (5 H, m, phenyl), 7.81 (1 H, s, H(7')).

El MS m/z: 356 (M+), 324, 296. Exact mass calculated for C\textsubscript{20}H\textsubscript{20}O\textsubscript{6}: 356.1260; found: 356.1268.

4.4. (±)-2-(3-BENZYL\textsubscript{OXY}-4-METHOXYBENZYL)BUTANEDIOIC ACID 1-METHYL ESTER (70)

Hemisuccinate 69 (96.4 g, 271 mmol) was added to a suspension of magnesium shavings (66.2 g, 2.72 mol) in dry methanol (750 mL) under nitrogen. After a few minutes stirring, the reaction vessel was immersed in an ice bath and stirred at 0°C for 5 h. The suspension was acidified with 6 M hydrochloric acid and the remaining solids were removed by filtration. The filtrate was extracted with dichloromethane (3 x 400 mL), washed with brine (500 mL) dried over magnesium sulfate, filtered and evaporated in vacuo to give the hemisuccinate 70 (86.4 g, 89%) as an amber resin which solidified upon standing at 3°C.

Physical properties of 70

Mp 78-82°C
IR ν\textsubscript{max} (CH\textsubscript{3}Cl): 1720 cm\textsuperscript{-1}.
UV λ\textsubscript{max} (log ε): 279 (3.49) nm.
\textsuperscript{1}H-NMR (300 MHz, CDCl\textsubscript{3}) δ: 2.34 (1 H, dd, H(3), J = 18, 4.5 Hz), 2.58-2.72 (2 H, m, H(3), H(2)), 2.90-3.10 (2 H, m, H(7')), 3.66 (3 H, s, -CO\textsubscript{2}H\textsubscript{3}), 3.88 (3 H, s, Ar-OC\textsubscript{6}H\textsubscript{5}), 5.13 (2 H, s, -OCH\textsubscript{2}Ph), 6.70 (2 H, m, H(2'), H(6')), 6.82 (1 H, d, H(5'), J = 7.8 Hz), 7.25-7.50 (5 H, m, phenyl).
El MS m/z: 358 (M⁺), 308, 281. Exact mass calculated for C₂₀H₂₂O₆: 358.1417; found: 358.1413.

4.5. (±)-3-(3-BENZYOXY-4-METHOXYBENZYL)BUTANOLIDE (71)

![Chemical Structure](image)

4.5.1. METHOD 1

The hemisuccinate 70 (2.1 g, 5.7 mmol) was dissolved at 0° C in water (5 mL) with 2 M potassium hydroxide. The solution was neutralized and evaporated in vacuo to yield the potassium salt of 70. A solution of calcium borohydride was prepared by adding a solution of calcium chloride (3.29 g, 28.5 mmol) in methanol (60 mL) to a solution of sodium borohydride (1.62 g, 40.7 mmol) in ethanol (60 mL) at -20° C. Potassium hydroxide (180 mg, 3.2 mmol) was added followed by the potassium salt of 70 dissolved in ethanol (50 mL). The solution was allowed to warm to room temperature and stirred for 4.5 d before being cooled to 0° C and acidified with 6 M hydrochloric acid. The solution was concentrated in vacuo, extracted with dichloromethane (3 x 100 mL), dried over magnesium sulfate, filtered and evaporated in vacuo. Purification by flash chromatography using ethyl acetate/petroleum ether (9:11, v/v) gave the 8-benzylbutanolide 71 (1.16 g, 65%) as a yellowish oil which solidified upon standing.

4.5.2. METHOD 2

Lithium borohydride (0.91 g, 42 mmol) in dry THF (150 mL) was added carefully to a solution of the hemisuccinate 70 (14.7 g, 40.9 mmol) in THF (250 mL) at reflux under nitrogen. The solution was stirred for 2 h at reflux and then cooled to room temperature. Water (2 mL) and 6 M hydrochloric acid (9 mL) were added and the solution was stirred at room temperature for 2.5 h. The bulk of the solvent was removed by evaporation in vacuo and the resultant mixture extracted with ether (50 mL). The organic
extract was washed with saturated sodium bicarbonate (3 x 25 mL) and water (25 mL) before being dried over magnesium sulfate, filtered and evaporated in vacuo to give the pure β-butanolide 71 (11.4 g, 88%) as a clear oil which solidified upon standing.

Physical properties of 71

\[ \text{Mp} 51-54^\circ \text{C} \]
\[ \text{IR} \nu_{\max} (\text{CHCl}_3): 1770 \text{ cm}^{-1}. \]
\[ \text{UV} \lambda_{\max} (\log \varepsilon): 279 \text{ (3.48) nm}. \]
\[ ^1\text{H-NMR} (400 \text{ MHz}, \text{CHCl}_3) \delta: 2.19 (1 \text{ H, dd, H(2), } J = 17.6, 6.7 \text{ Hz}), 2.50 (1 \text{ H, dd, H(2), } J = 17.6, 7.9 \text{ Hz}), 2.61-2.80 (3 \text{ H, m, H(3), H(7')}), 3.89 (3 \text{ H, s, } -\text{OCH}_3), 3.91 (1 \text{ H, dd, H(4), } J = 9.1, 6.0 \text{ Hz}), 4.21 (1 \text{ H, dd, H(4), } J = 9.1, 7.0 \text{ Hz}), 5.16 (2 \text{ H, s, } -\text{OCH}_2\text{Ph}), 6.65 (1 \text{ H, d, H(2'), } J = 1 \text{ Hz}), 6.71 (1 \text{ H, dd, H(6')}, J = 8, 1 \text{ Hz}), 6.85 (1 \text{ H, d, H(5')}, J = 8 \text{ Hz}). \]
\[ \text{EI MS } m/z: 312 \text{ (M+), 222, 137. Exact mass calculated for C}_{19}\text{H}_{20}\text{O}_4: 312.1362; \text{ found: 312.1371}. \]

4.6. \((\pm)-\text{TRANS-2-(4-BENZYLOXY-3,5-DIMETHOXYBENZYL)-3-(3-BENZYLOXY-4-METHOXYBENZYL)BUTANOLIDE (73)}\)

![Diagram of compound 73]

To a solution of diisopropylamine (2.9 mL, 21 mmol) in dry THF (30 mL) at -78° C was added butyllithium (11 mL 1.6 M solution, 17 mmol) and stirring continued for 15 min at -78° and then for 30 min at 0° C. The clear solution was cooled to -78° C, β-butanolide 71 (4.3 g, 14 mmol) in THF (15 mL) was added and the
bright yellow solution stirred for 90 min prior to the addition of the bromide 72 (5.5 g, 16 mmol) in THF (15 mL) and stirring for 8 h at -78° C. The solution was warmed to 0° C, acidified with 1 M hydrochloric acid and extracted with dichloromethane (2 x 150 mL). The combined organic extracts were washed with water (80 mL), dried over magnesium sulfate, filtered and evaporated in vacuo. Flash chromatography using ethyl acetate/petroleum ether (9:11, v/v) gave the dibenzylbutanolide 73 (6.2 g, 80%) as a yellowish foam.

Physical properties of 73

Mp 35-37° C
IR νmax (CHCl3): 1770 cm⁻¹.
UV λmax (log ε): 278 (3.55) nm.

¹H-NMR (400 MHz, CHCl3) δ: 2.32 (1 H, dd, H(7"), J = 14.4, 8.2 Hz), 2.45-2.70 (3 H, m, H(7"), H(2), H(3)), 3.06 (1 H, dd, H(7'), J = 14.0, 8.0 Hz), 3.32 (1 H, dd, H(7'), J = 14.0, 5.4 Hz), 3.74 (1 H, dd, H(4), J = 9, 8 Hz), 3.80 (6 H, s, -OCH3(3",5")), 3.88 (3 H, s, -OCH3(4")), 3.99 (1 H, dd, H(4), J = 9.2, 7.0 Hz), 5.05, 5.13 (2 H each, s, s, -OCH2Ph), 6.52-6.58 (3 H, m, H(2"), H(6")), 6.69 (1 H, s, H(2')), 6.77 (1 H, d, H(5'), J = 8.0 Hz), 7.25-7.49 (10 H, m, phenyl).

El MS m/z: 568 (M⁺), 402, 312, 167, 137, 91. Exact mass calculated for C₃₅H₃₆O₇: 568.2461; found: 568.2457.
4.7. (±)-TRANS-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)BUTANOLIDE (58)

Palladium on charcoal (5%, 1.05 g) was suspended in ethanol (40 mL) and stirred under hydrogen at atmospheric pressure for 1.5 h. The dibenzylbutanolide 73 (6.2 g, 11 mmol) was added as an ethyl acetate/ethanol solution (1:3, v/v, 40 mL) and the resultant suspension stirred for 5 h. The catalyst was filtered off and the solvent evaporated in vacuo to yield the bis(hydroxybenzyl)butanolide 58 (3.7 g, 87%) as an amorphous white solid after flash chromatography using ethyl acetate/petroleum ether (11:9, v/v). Small samples were crystallized from diethyl ether/petroleum ether for melting point determination or distilled (240°C, 0.15 mm) for microanalysis.

Physical properties of 58

Mp 68-69°C (diethyl ether/petroleum ether)

IR $\tilde{\nu}_{\text{max}}$ (CHCl$_3$): 3540, 1770 cm$^{-1}$.

UV $\lambda_{\text{max}}$ (log $\varepsilon$): 279 (3.57) nm.

$^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 2.45-2.65 (4 H, m, H(2), H(3), H(7*)), 2.88 (1 H, dd, H(7'), J = 14.6, 5.7 Hz), 2.93 (1 H, dd, H(7'), J = 14.6, 6.4 Hz), 3.87-3.89 (10 H, m, -OCH$_3$, H(4)), 4.15 (1 H, dd, H(4), J = 7.5, 3.0 Hz), 5.42 (1 H, s, -OH(4*)), 5.58 (1 H, s, -OH(3*)), 6.49 (1 H, dd, H(6'), J = 8.5, 2.1 Hz), 6.62 (1 H, d, H(2'), J = 2.1 Hz), 6.73 (1 H, d, H(5'), J = 8.5 Hz). All hydroxyl signals disappear upon addition of D$_2$O.

$^{13}$C-NMR (75.3 MHz, acetone-d$_6$) $\delta$: 35.363 (C(7*)), 38.147 (C(7*)), 41.891(-), 47.055(-), 56.175(-),
To a solution of piperonal (32.1 g, 6.7 mmol) in dry chloroform (10 mL) at -40° C under argon was added thiophenol (1.4 mL, 14 mmol) and boron trifluoride etherate (4.6 mL 45% solution, 17 mmol). The solution was stirred for 15 min at -40° C and then poured into ice-cold water (10 mL). The organic layer was drawn off and the aqueous layer extracted with chloroform (30 mL). The combined organic extracts were washed with 7% potassium hydroxide (2 x 25 mL), water (25 mL) and brine (25 mL) before being dried over potassium carbonate, filtered and evaporated in vacuo to yield the pure dithioacetal 97 (2.3 g, 98%) as a yellowish oil. The physical properties of 97 were determined using a distilled (190° C, 0.15 mm) sample.

Physical properties of 97

IR $\nu_{\text{max}}$ (neat): 2890, 1490 cm$^{-1}$.

UV $\lambda_{\text{max}}$ (log $\varepsilon$): 248 (4.14), 282 (3.88) nm.

$^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 5.37 (1 H, s, -CH(SPh)$_2$), 5.96 (2 H, s, -OCH$_2$O-), 6.67 (1 H, d, H(5), J = 4 Hz), 6.78 (1 H, dd, H(6), J = 4, 2 Hz), 6.98 (1 H, d, H(2), J = 2 Hz), 7.23-7.32 (6 H, m, phenyl), 7.34-7.42
(4 H, m, phenyl).

El MS m/z: 352 (M+), 243. Exact mass calculated for C\textsubscript{20}H\textsubscript{16}O\textsubscript{2}S\textsubscript{2}: 352.0592; found: 352.0598.

Elemental analysis: calculated for C\textsubscript{20}H\textsubscript{16}O\textsubscript{2}S\textsubscript{2}: C (68.15), H (4.58); found: C (68.11), H (4.63).

4.9. (±)-TRANS-2-(4-BENZYOXY-3,5-DIMETHOXYBENZYL)-3-((3,4-METHYLENE-DIOXY)-α,α-BIS(PHENYLTHIO)BENZYL)BUTANOLIDE (96)

Butyllithium (3.60 mL 1.6 M solution, 5.76 mmol) was added to a solution of dithioacetal 97 (2.00 g, 5.69 mmol) in dry THF (10 mL) at -78° C and the purple solution was stirred for 2.5 h. But-2-en-4-olide (98, 0.481 g, 5.73 mmol) in THF (8 mL) was added and the solution stirred for 2.5 h at -78° C prior to the addition of the bromide 72 (2.06 g, 6.10 mmol) in THF (10 mL). The solution was allowed to warm slowly to room temperature overnight. Water (25 mL) was added and the mixture extracted with ethyl acetate (160 mL). The organic extract was washed with water (2 x 25 mL) before being dried over magnesium sulfate, filtered and evaporated in vacuo. Purification by flash chromatography using dichloromethane gave the dithioketal 95 (2.16 g, 55%) as an amorphous solid. A sample was further purified for microanalysis by recrystallization from acetone.
Physical properties of 96

Mp 55-57° C (acetone)

IR $\nu_{\text{max}}$ (CHCl$_3$): 1770 cm$^{-1}$.

UV $\lambda_{\text{max}}$ (log e): 279 (3.82), 286 (3.80) nm.

$^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 2.79 (1 H, dd, H(7$''$), $J = 14$, 5 Hz), 2.85-2.90 (1 H, m, H(2)), 3.12 (1 H, dd, H(7$''$), $J = 14$, 4 Hz), 3.29-3.34 (1 H, m, H(3)), 3.49 (1 H, dd, H(4), $J = 10$, 8 Hz), 3.70 (6 H, s, -OCH$_3$), 4.46 (1 H, dd, H(4), $J = 10$, 3 Hz), 5.02 (2 H, s, -OCH$_2$Ph), 6.00, 6.01 (2 H total, d, d, -OCH$_2$O-, $J = 1$ Hz each), 6.18 (2 H, s, H(2$''$)), 6.73 (1 H, d, H(5$''$), $J = 8$ Hz), 7.13 (1 H, dd, H(6$''$), $J = 8$, 2 Hz), 7.21-7.38 (14 H, m, phenyl, H(2$''$)), 7.43-7.47 (2 H, m, phenyl).

FAB MS m/z: 693 (M$^+$), 584, 473, 383, 218, 185, 167, 154, 135.

EI MS m/z: 584, 583, 383, 218, 185, 167, 154, 135. Exact mass calculated for C$_{34}$H$_{31}$O$_7$S (M$^+$ - SPh): 583.1790; found: 583.1792.

Elemental analysis: calculated for C$_{40}$H$_{36}$O$_7$S$_2$: C (69.34), H (5.24), S (9.25); found: C (69.18), H (5.42), S (9.23).
4.10. (±)-TRANS-2-(4-BENZYOXY-3,5-DIMETHOXYBENZYL)-3-(3,4-METHYLENE-DIOXYBENZOYL)BUTANOLIDE (100) AND (±)-TRANS-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3,4-METHYLENEDIOXYBENZOYL)BUTANOLIDE (101)

4.10.1. METHOD 1

Dithioketal 96 (34.5 mg, 50.0 µmol) was dissolved in ethyl acetate (6 mL). Perchloric acid (70%, 1 drop) was added and the solution stirred for 4 h. Water (1 mL) and ethyl acetate (10 mL) were added and the phases separated. The organic extract was washed with brine (2 mL) and saturated sodium bicarbonate (2 x 2 mL), dried over magnesium sulfate, filtered and evaporated in vacuo. Flash chromatography using acetone/dichloromethane (1:19, v/v) gave the ketones 100 (10.8 mg, 44%) and 101 (2.0 mg, 10%).

4.10.2. METHOD 2

Sulfonyl chloride (11.8 mg, 87.4 µmol) in dichloromethane (0.4 mL) was added to a mixture of dithioketal 96 (48.7 mg, 70.3 µmol), silica gel (230-400 mesh, 30 mg) and water (30 mg) in dichloromethane (0.35 mL). After stirring for 2 h, finely powdered anhydrous potassium carbonate was added and stirring continued for 0.5 h. The solids were filtered off and washed with dichloromethane. The filtrate was evaporated in vacuo and purified by flash chromatography using acetone/dichloromethane (1:49, v/v) but neither 100 nor 101 could be identified by ¹H-NMR spectroscopy of the major fractions.
4.10.3. METHOD 3

Iodine (1.46 g, 11.5 mmol) was added to a solution of dithioketal 96 (1.85 g, 2.68 mmol) in methanol (50 mL) and THF (5 mL). The solution was then stirred at reflux for 1.5 h prior to the addition of saturated sodium thiosulfate (10 mL), brine (10 mL) and water (20 mL). The mixture was extracted with ethyl acetate (100 mL), diluted with water (10 mL) and brine (10 mL) and re-extracted with ethyl acetate (100 mL). The organic extract was washed with water (20 mL), dried over magnesium sulfate, filtered and evaporated \textit{in vacuo}. THF (5 mL) and 3 M hydrochloric acid (6 mL) were added and the suspension stirred at room temperature for 1 h before being diluted with water (10 mL) and extracted with dichloromethane (2 x 50 mL). The organic extract was washed with water (2 x 20 mL), dried over magnesium sulfate, filtered and evaporated \textit{in vacuo}. Ether (25 mL) was added and the suspension sonicated for 5 min, stored at 3° C for 3 d, filtered and washed with ice-cold ether (2 x 5 mL) to give the ketone 100 as a white powder (0.920 g). The filtrate was evaporated and purified by flash chromatography using acetone/dichloromethane (0:1 to 1:49 gradient, v/v) to afford 100 (134 mg) for a total yield of 80%.

Physical properties of 100

Mp 43-44° C (diethyl ether)

IR $\nu_{\text{max}}$ (CHCl$_3$): 1770, 1670 cm$^{-1}$.

UV $\lambda_{\text{max}}$ (log $e$): 275 (3.82), 314 (3.90) nm.

$^1$H-NMR (400 MHz, CHCl$_3$) $\delta$: 3.00 (1 H, dd, H(7$''$), J = 14, 6 Hz), 3.09 (1 H, dd, H(7$''$), J = 14, 5 Hz), 3.55-3.61 (1 H, m, H(2)), 3.70 (6 H, s, -OCH$_3$), 4.01 (1 H, ddd, H(3), J = 8, 8, 8 Hz), 4.10 (1 H, dd, H(4), J = 9, 8 Hz), 4.38 (1 H, dd, H(4), J = 9, 8 Hz), 4.92 (2 H, s, -OCH$_2$Ph), 6.01, 6.04 (1 H each, d, d, -OCH$_2$O-, J = 1 Hz each), 6.28 (2 H, s, H(2$''$)), 6.80 (1 H, d, H(5$'$), J = 8 Hz), 7.24-7.38 (5 H, m, H(2$'$), H(6$'$), phenyl), 7.47 (2 H, dd, phenyl, J = 7.1 Hz).

El MS m/z: 490 (M$^+$), 399, 223, 149, 91. Exact mass calculated for C$_{28}$H$_{26}$O$_8$: 490.1627; found: 490.1627.

Elemental analysis: calculated for C$_{28}$H$_{26}$O$_8$: C (68.56), H (5.34); found: C (68.78), H (5.56).
Physical properties of 101

Mp 56-58° C

IR $\nu_{\text{max}}$ (CHCl$_3$): 3530, 1770, 1670 cm$^{-1}$.

UV $\lambda_{\text{max}}$ (log $\varepsilon$): 276 (3.80), 314 (3.86) nm.

$^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 3.01 (1 H, d, H(7$''$), J = 14, 7 Hz), 3.07 (1 H, dd, H(7$''$), J = 14, 6 Hz), 3.53-3.60 (1 H, m, H(2)), 3.78 (6 H, s, -OCH$_3$), 4.02 (1 H, ddd, H(3), J = 9, 9, 9 Hz), 4.12 (1 H, dd, H(4), J = 9, 9 Hz), 4.41 (1 H, dd, H(4), J = 9, 9 Hz), 5.37 (1 H, s, -OH, disappears with D$_2$O), 6.07 (2 H, m, -OCH$_2$O$^-$), 6.30 (2 H, s, H(2$''$)), 6.81 (1 H, d, H(5'), J = 8 Hz), 7.23 (1 H, d, H(2'), J = 2 Hz), 7.27 (1 H, dd, H(6'), J = 8, 2 Hz).

EI MS m/z: 400 (M$^+$), 224, 167, 149. Exact mass calculated for C$_{21}$H$_{20}$O$_8$: 400.1158; found: 400.1164.

4.11. (±)-TRANS-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(α-HYDROXY-3,4-METHYLENEDIOXYBENZYL)BUTANOLIDE (102)
4.11.1. METHOD 1

Palladium on charcoal (10%, 1.676 g) was suspended in ethanol (100 mL) and stirred for 1 h under hydrogen at atmospheric pressure. A solution of the ketone 100 (4.50 g, 9.16 mmol) in ethyl acetate (100 mL) was added and the resultant suspension stirred for 7 h. The catalyst was filtered off and the solution evaporated in vacuo. The residue was purified by column chromatography using acetone/dichloromethane (1:19 to 1:9 gradient, v/v) to give ketone 100 (0.685 g, 15%), ketone 101 (1.686 g, 46%) and alcohol 102 (1.113 g, 30%, 3.3:1 mixture of C(7) epimers according to $^1$H-NMR spectroscopy).

4.11.2. METHOD 2

Sodium borohydride (47 mg, 1.2 mmol) was added to a suspension of the ketone 101 (464 mg, 1.16 mmol) in dry methanol (50 mL) at 0° C under nitrogen. After stirring for 2.5 h at 0° C, 1 M hydrochloric acid (2 mL) was added to the resultant clear solution, which was then concentrated in vacuo, diluted with water (5 mL) and brine (5 mL) and extracted with ethyl acetate (2 x 50 mL). The combined organic extracts were washed with water (15 mL), dried over magnesium sulfate, filtered and evaporated in vacuo to give the alcohol 102 (443 mg, 95%) as an amorphous white solid. $^1$H-NMR spectroscopy showed the product to be a 6:1 mixture of C(7) epimers.

Properties of 3.3:1 102 mixture (C(7) β hydroxyl epimer assumed as major)

IR $\tilde{\nu}_{\text{max}}$ (CHCl$_3$): 3800, 3530, 1770 cm$^{-1}$.

UV $\lambda_{\text{max}}$ (log e): 282 (3.60) nm.

$^1$H-NMR (400 MHz, CDCl$_3$, integrals relative to minor (α) epimer) δ: 1.94 (1 H, d, -OH(7',α), J = 3 Hz), 2.00 (3.3 H, d, -OH(7',β), J = 3 Hz), 2.51-3.11 (17.2 H, m, H(7''), H(2), H(3)), 3.85 (6 H, s, -OCH$_3$(α)), 3.87 (19.8 H, s, -OCH$_3$(β)), 3.92-3.96 (6.6 H, m, H(4,8)), 4.17 (1 H, dd, H(4,α), J = 9.6, 7.6 Hz), 4.37 (1 H, dd, H(7',α), J = 8.0, 2.5 Hz), 4.40 (1 H, dd, H(4,β), J = 9.6, 6.1 Hz), 4.62 (3.3 H, dd, H(7',β), J = 6.7, 2.9 Hz), 5.40 (4.3 H, s, -OH(4'')), 5.97, 5.98 (3.3 H each, d, d, -OCH$_2$O-(β), J = 1 Hz), 5.99, 6.00 (1 H each, d, d, -OCH$_2$O-(α), J = 1 Hz), 6.27 (2 H, s, H(2'',α)), 6.40 (6.6 H, s, H(2'',β)), 6.61-6.76 (12.9 H, m, H(5''), H(5'), H(6')). Upon addition of D$_2$O, all hydroxyl signals disappear and the H(7') signals collapse to
doublets.

EI MS m/z: 402 (M+), 384, 178, 167, 224, 151, 135, 123. Exact mass calculated for C_{21}H_{20}O_8: 402.1314; found: 402.1302.

Elemental analysis: calculated for C_{21}H_{22}O_8: C (62.68), H (5.51); found: C (62.33), H (5.80).

Physical properties of 6:1 102 mixture

Mp 43-51° C

4.12. \((\pm)-\text{TRANS}-2-(3,5\text{-DIMETHOXY}-4\text{-HYDROXYBENZYL})-3-(3,4\text{-DIHYDROXY}-\alpha\text{-HYDROXYBENZYL})\text{BUTANOLIDE (94) AND (\pm)-4-(3,4\text{-DIHYDROXYPHENYL}-5,7\text{-DIMETHOXY}-6\text{-HYDROXY}-2\text{-HYDROXYMETHYL}-1,2,3,4\text{-TETRAHYDRO}-2\text{-NAPHTHOIC ACID }\gamma\text{ LACTONE (103)}}\)

4.12.1. \textsc{Method 1}

Alcohol 102 (210 mg, 0.52 mmol) dissolved in dichloromethane (10 mL) was added to a solution of boron trichloride (3.1 mmol) in dichloromethane (23.5 mL) at -78° C. After stirring for 2 h at -78° C, saturated potassium bicarbonate (2 mL) was added and the suspension allowed to warm to room temperature. Water (5 mL) and brine (1 mL) were added and the mixture was extracted with ethyl acetate
(2 x 50 mL). The organic extract was dried over magnesium sulfate, filtered and evaporated in vacuo. Acetone (4 mL), water (5 mL) and calcium carbonate (330 mg) were added and the suspension stirred for 1.5 h at 65°C. The solids were dissolved by the addition of 6 M hydrochloric acid and the clear solution extracted with ethyl acetate (2 x 50 mL). The organic extract was washed with water (15 mL), dried over magnesium sulfate, filtered and evaporated in vacuo. Flash chromatography using acetone/dichloromethane (2:8 to 3:8, v/v) gave the aryltetralin 103 (78 mg, 40%) and the catechol 94 (49 mg, 24%, single epimer by 1H-NMR spectroscopy) as amorphous white solids. A sample of 103 was recrystallized from methanol/chloroform for analysis.

4.12.2. METHOD 2

Alcohol 109 (161 mg, 0.327 mmol) dissolved in dichloromethane (6.5 mL) was added to a stirred solution of boron trichloride (2.0 mmol) in dichloromethane (15.3 mL) at -78°C. After stirring for 2 h, water (3 mL), saturated potassium hydrogen carbonate (1 mL) and brine (1 mL) were added and the mixture allowed to warm to room temperature. The mixture was extracted with ethyl acetate (2 x 40 mL), dried over magnesium sulfate, filtered and evaporated in vacuo. Acetone (3 mL), water (6.5 mL) and calcium carbonate (210 mg) were added and the suspension stirred for 1.5 h at 65°C. The solids were dissolved with 6 M hydrochloric acid and the solution extracted with ethyl acetate (2 x 40 mL). The organic extracts were washed with water (10 mL), dried over magnesium sulfate, filtered and evaporated in vacuo to afford a yellowish foam. Flash chromatography using acetone/dichloromethane (1:4 to 3:2 gradient, v/v) afforded the alcohol 102 (42 mg, 32%, 5:1 mixture of epimers by 1H-NMR spectroscopy) and the catechol 94 (33 mg, 26%).

Physical properties of 94

Mp 49-56°C
IR \( \nu_{\text{max}} \) (Nujol): 3380, 1740 cm\(^{-1}\).
UV \( \lambda_{\text{max}} \) (log e): 281 (3.57) nm.

1H-NMR (400 MHz, acetone-d\(_6\)) \( \delta \): 2.64-2.77, 2.95-3.05 (4 H total, m, m, H(7"), H(2), H(3)), 3.80 (6 H, s, -
Upon addition of D$_2$O, all hydroxyl signals disappear and the H(7') signal collapses to a doublet.

$^{13}$C-NMR (300 MHz, acetone-d$_6$): 35.619 (C(7'')), 43.787(-), 45.709(-), 56.464(-) (-$^{18}$OCH$_3$), 68.892 (C(4'')), 74.217(-) (C(7'')), 107.994(-) (C(2'')), 114.086(-), 115.787(-), 118.432(-), 129.114, 135.743, 145.227, 145.826, 148.339, 179.568 (C(1)).

El MS m/z: 390 (M$^+$), 372, 167, 154, 123, 110. Exact mass calculated for C$_{20}$H$_{22}$O$_8$: 390.1315; found: 390.1313.

Physical properties of 103

Mp 222-224°C (methanol/chloroform)

IR $\nu_{\text{max}}$ (Nujol): 3400, 3250, 1730 cm$^{-1}$.

UV $\lambda_{\text{max}}$ (log $\varepsilon$): 283 (3.70) nm

$^1$H-NMR (400 MHz, acetone-d$_6$): 2.30-2.41 (1 H, m, H(3)), 2.51 (1 H, ddd, H(2), J = 13.6, 11.7, 4.5 Hz), 2.88-2.92 (1 H, m, H(1)), 3.03 (1 H, dd, H(1), J = 15.3, 4.5 Hz), 3.18 (3 H, s, -OCH$_3$(5)), 3.87 (3 H, s, -OCH$_3$(7)), 4.04 (1 H, br d, H(4), J = 10.8 Hz), 4.16-4.20 (2 H, m, H(11)), 6.50 (1 H, dd, H(6'), J = 8, 2 Hz), 6.55 (1 H, d, H(2'), J = 2 Hz), 6.65 (1 H, s, H(8)), 6.74 (1 H, d, H(5'), J = 8 Hz), 7.12 (1 H, s, -OH(6)), 7.54, 7.56 (1 H each, s, s, -OH(3'), -OH(4')). All hydroxyl signals disappear upon addition of D$_2$O.

$^{13}$C-NMR (50.2 MHz, acetone-d$_6$): 41.595(-), 46.525(-), 50.499(-), 56.255(-), 58.614(-), 72.246, 101.389, 107.912(-), 114.565(-), 115.779(-), 118.991(-), 127.733, 139.071, 139.320, 143.913, 145.686, 177.2 (C(1)).

El MS m/z: 372 (M$^+$), 262. Exact mass calculated for C$_{20}$H$_{20}$O$_7$: 372.1219; found: 372.1217.

Elemental analysis: calculated for C$_{20}$H$_{20}$O$_7$: C (64.51), H (5.41); found: C (63.58), H (5.57).
4.13. (±)-5,7-DIMETHOXY-6-HYDROXY-3-HYDROXYMETHYL-4-(3,4-METHYLENE-DIOXYPHENYL)-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID γ LACTONE (104)

Alcohol 102 (67 mg, 0.17 mmol) in dichloromethane (8 mL) was added to a stirred solution of boron trichloride (0.25 mmol) in dry dichloromethane (5.25 mL) at -100° C. The pale yellow solution was stirred for 1 h at -100° C before water (6 mL) and saturated potassium bicarbonate (3 mL) were added. The mixture was extracted with ethyl acetate (2 x 40 mL) and the organic extract dried over magnesium sulfate, filtered and evaporated *in vacuo*. Acidification of the residual aqueous phase with 6 M hydrochloric acid and extraction with ethyl acetate (40 mL) gave, after drying the organic extract over magnesium sulfate, filtering and evaporating *in vacuo*, an insignificant amount of material (less than 1 mg). Flash chromatography using acetone/dichloromethane (1:9, v/v) afforded alcohol 102 (46 mg, 69%) and aryltetralin 104 (4 mg, 6%).

Physical properties of 104

Mp 196-197° C
IR $\tilde{\nu}_{\text{max}}$ (CHCl$_3$): 3540, 1775 cm$^{-1}$.
UV $\lambda_{\text{max}}$ (log ε): 285 (3.71) nm.
$^1$H-NMR (400 MHz, CDCl$_3$) δ: 2.29-2.47 (2 H, m, H(2), H(3)), 2.95 (1 H, br dd, H(1), J = 15, 11 Hz), 3.15 (1 H, dd, H(1), J = 15, 4 Hz), 3.29 (3 H, s, -OCH$_3$(5)), 3.91 (3 H, s, -OCH$_3$(7)), 4.01 (1 H, d, H(4), J = 10
Hz), 4.08 (1 H, dd, H(11)), J = 10, 8 Hz), 4.30 (1 H, dd, H(11)), J = 10, 6 Hz), 5.38 (1 H, s, -OH(6), disappears upon addition of D₂O), 5.93 (2 H, s, -OCH₂O⁻), 6.51 (1 H, s, H(8)), 6.52 (1 H, br s, H(2')), 6.58 (1 H, br d, H(6'), J = 8 Hz), 6.74 (1 H, d, H(5'), J = 8 Hz).

EI MS m/z: 384 (M⁺), 262, 178, 135. Exact mass calculated for C₂₁H₂₀O₇: 384.1209; found: 384.1213.

4.14. (±)-TRANS-2-(4-BENZYL-OXY-3,5-DIMETHOXYBENZYL)-3-(α-HYDROXY-3,4-METHYLENEDIOXYBENZYL)BUTANOLIDE (109)

4.14.1. METHOD 1

Sodium borohydride (19 mg, 0.51 mmol) was added to a suspension of ketone 100 (173 mg, 0.352 mmol) in dry methanol (25 mL) at 0°C under nitrogen. After stirring for 5 h at 0°C, 1 M hydrochloric acid (3 mL) was added to the resultant clear solution, which was then concentrated in vacuo, diluted with brine (2 mL) and extracted with dichloromethane (2 x 40 mL). The combined organic extracts were washed with water (8 mL), dried over magnesium sulfate, filtered and evaporated in vacuo to give alcohol 109 (172 mg, 99%) as an amorphous white solid. ¹H-NMR spectroscopy indicated that the product was a 4.9:1 mixture of epimers.
4.14.2. METHOD 2

Lithium aluminum tri-t-butoxy hydride (690 mg, 2.7 mmol) in THF (8 mL) was added to a stirred suspension of the ketone 100 (380 mg, 0.78 mmol) in dry ether (25 mL) and THF (5 mL) under nitrogen at -78° C. The mixture was stirred for 3 h and then acidified with 1 M hydrochloric acid (6 mL), diluted with water (15 mL) and extracted with ethyl acetate (2 x 50 mL). The organic extract was washed with saturated sodium bicarbonate (2 x 15 mL) and water (10 mL), dried over magnesium sulfate, filtered and evaporated in vacuo to afford a foam (329 mg) which, by 1H-NMR spectroscopy, contained the alcohol 109 (70%, 5.7:1 mixture of epimers) and the ketone 100 (30%).

Physical properties of 4.9:1 109 mixture (C(7) β hydroxyl epimer assumed as major)

Mp 46-49°

IR νmax (CHCl3): 3450, 1770 cm\(^{-1}\).

UV λmax (log ε): 284 (3.55) nm.

1H-NMR (300 MHz, CDCl₃, integrals relative to minor (α) epimer) δ: 1.87 (1 H, d, OH(7,α), J = 3 Hz), 1.96 (4.9 H, d, -OH(7,β), J = 2.8 Hz), 2.47-3.10 (23.6 H, m, H(2), H(3), H(7″)), 3.78-3.79 (35.4 H, m, -OCH₃), 3.89-3.96 (9.8 H, m, H(4,β)), 4.15-4.17 (1 H, m, H(4,α)), 4.33 (1 H, dd, H(7,α), J = 7.1, 3 Hz), 4.37 (1 H, dd, H(4,α), J = 9.3, 6.4 Hz), 4.60 (4.9 H, dd, H(7,β), J = 7.1, 2.8 Hz), 5.00 (11.8 H, s, -OCH₂Ph), 5.93-6.01 (11.8 H, m, -OCH₂O⁻), 6.25 (2 H, s, H(2,α)), 6.38 (9.8 H, s, H(2,β)), 6.57-6.78 (17.7 H, m, H(2′), H(5′), H(6′)), 7.22-7.50 (29.5 H, m, phenyl). Upon D₂O addition, both hydroxyl signals disappear and H(7′) signals collapse to doublets.

El MS m/z: 492 (M⁺), 474, 161, 149, 131, 91. Exact mass calculated for C₂₈H₂₈O₈: 492.1784; found: 492.1784.
4.15. PREPARATION OF C. ROSEUS CELL-FREE EXTRACT (CFE)

*C. roseus* (AC 3 line) cell suspension culture was grown in shake flasks in the dark in a 1B5 medium\(^{124}\) (500 mL) containing agar (7-8 g) at pH 5.5. The cell line was subcultured every 10 d using a 12% (60 mL) inoculum and harvested at 11 d age. The whole cell suspension was filtered with Microcloth, drained thoroughly, washed with water (75 mL/flask) and drained again. The rest of the procedure was carried out at 0-4° C. Potassium phosphate buffer (0.100 M, pH \(pH_1\), 0.50 mL/g fresh weight) was added and the suspension homogenized with an Ultra-Turrax blender at 20,000 rpm in four 30 s periods with 30 s intervals. The material was centrifuged, using a Sorvall RC-5B centrifuge and Sorvall GSA rotor, at 10,000 g for 30 min and the supernatant (CFE) analyzed for peroxidase activity\(^{96}\) and protein concentration\(^{101,102}\) using a Bausch and Lomb Spectronic 20 spectrophotometer.

4.16. SMALL SCALE BIOTRANSFORMATION OF *TRANS*-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)BUTANOLIDE (58)

An aliquot (3.00 mL) of a solution of 58 (15.3 mg, 39.3 \(\mu\)mol) in ethanol/water (8:17, v/v) was added to water and 81.6 mM hydrogen peroxide (4.00 mL combined volume). Potassium phosphate buffer (0.100 M) and *C. roseus* CFE (18.0 mL combined volume) were added and the solution was stirred for 3 h. Both the buffer and CFE were adjusted to the appropriate value of pH prior to the biotransformation. Reaction samples (1.00 mL) were quenched with methanol (1.00 mL) and filtered through a cotton plug prior to HPLC analysis.
4.17. DETERMINATION OF HYDROGEN PEROXIDE CONSUMPTION BY C. ROSEUS CFE

An aliquot (3.00 mL) of a solution of 58 (15.3 mg, 39.3 μmol) in ethanol/water (8:17, v/v) was added to water (3.00 mL) and hydrogen peroxide (1.00 mL, 81.6 mM solution, 81.6 μmol). Potassium phosphate buffer (0.100 M, pH 6.3, 10.70 mL) and CFE (pH 6.3, specific activity 0.98 units/mg, 7.30 mL, 9.83 units) were added and the solution (Solution A) stirred for 3 h. An identical solution of water, hydrogen peroxide, buffer and CFE was stirred for 15 min upon the addition of CFE: an aliquot of 58 was then added as before and the solution (Solution B) stirred for an additional 3 h. After each solution had been stirred 1 h following addition of 58, hydrogen peroxide (1.00 mL, 81.6 μmol) was added to Solution B and water (1.00 mL) to Solution A. HPLC analysis was performed as before.

4.18. EXTRACTION OF TRANS-2-(3,5-DIMETHOXY-4-HYDOXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)BUTANOLIDE (58) AND 2-(3,5-DIMETHOXY-4-HYDROXYBENZYLIDENE)-3-(3,4-METHYLENEDIOXYBENZYL)BUTANOLIDE (74) BIOTRANSFORMATION MIXTURES

The reaction mixture was quenched with dichloromethane and filtered through Celite. The phases were separated and the aqueous extracted repeatedly with dichloromethane and ethyl acetate, respectively. The organic extracts were dried over magnesium sulfate, filtered and evaporated in vacuo.
4.19. (±)-1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-6-HYDROXY-3-
HYDROXYMETHYL-7-METHOXY-1,2,3,4-TETRAHYDO-2-NAPHTHOIC ACID γ
LACTONE (59) (PRELIMINARY EXPERIMENT, SECTION 2.2)

\[ \text{\text{CH}_3O} \]
\[ \text{\text{CH}_3O} \]
\[ \text{\text{OH}} \]

C. roseus CFE (pH 6.3, specific activity 1.3 units/mg, 45 mL, 95 units) was added to a solution containing butanolide 58 (150 mg, 0.40 mmol), ethanol (15 mL), water (135 mL) and hydrogen peroxide (5.0 mL, 0.17 M solution, 0.85 mmol). Four additional portions of hydrogen peroxide (5.0 mL, 0.85 mmol each) were added at 15 min intervals. The solution was stirred for a further 1 h and then extracted by the standard procedure. The dichloromethane extract was purified by column chromatography using chloroform/ethyl acetate (1:0 to 0:1 gradient, v/v) to give 58 (15 mg, 10%) and 59 (87 mg, 58%).

Physical properties of 59

Mp 240-241° C (chloroform)

IR \( \nu_{\text{max}} \) (CHCl\(_3\)): 3530, 1778 cm\(^{-1}\).

UV \( \lambda_{\text{max}} \) (log \( \varepsilon \)): 282 (3.53) nm.

\(^1\)H-NMR (400 MHz, CDCl\(_3\)) \( \delta \): 2.47 (1 H, dd, H(2), \( J = 14, 11 \) Hz), 2.59 (1 H, m, H(3)), 2.90 (1 H, br dd, H(4), \( J = 15, 11 \) Hz), 2.97 (1 H, dd, H(4), \( J = 14, 5 \) Hz), 3.64 (3 H, s, -OCH\(_3\)(7)), 3.85 (6 H, s, -OCH\(_3\)(3')), 3.98 (1 H, dd, H(11), \( J = 11, 8 \) Hz), 4.07 (1 H, br d, H(1), \( J = 11 \) Hz), 4.52 (1 H, dd, H(11), \( J = 11, 7 \) Hz),
5.43, 5.51 (1 H each, s, s, -OH(4'), -OH(6)), 6.34, 6.70 (1 H each, s, s, H(5), H(8)), 6.45 (2 H, s, H(2')). Both hydroxyl signals disappear upon addition of D$_2$O.

$^{13}$C-NMR (75.3 MHz, acetone-d$_6$) δ: 32.661 (C(4)), 40.681(-), 47.037(-), 48.544(-), 56.106(-), 56.558(-)
(-OCH$_3$(3')), 71.384 (C(11)), 107.810(-) C(2'), 113.754(-), 115.696(-), 115.777(-), 176 (C(1)).

EI MS m/z: 386 (M$^+$), 232, 167, 154, 139. Exact mass calculated for C$_{21}$H$_{22}$O$_7$: 386.1366; found: 386.1366.

Elemental analysis: calculated for C$_{21}$H$_{22}$O$_7$: C (65.28), H (5.74); found : C (65.12), H (5.72).

Circular dichroism analysis: no Cotton effect observed for 0.208 mM methanol solution (250-320 nm).

X-ray diffraction analysis: sample crystallized from chloroform/dichloromethane (1:1, v/v) by slow evaporation.

### Selected X-ray Crystallographic Data

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4.20. \((\pm)-2-(3,5\text{-dimethoxy}-4\text{-hydroxybenzylidene})-3-(3,4\text{-methylenedioxybenzyl})\text{butanolide} \ (76)\)

\[
\text{IR } \tilde{\nu}_{\text{max}} (\text{CHCl}_3): \ 3570, \ 1745 \text{ cm}^{-1}.
\]

\[
\text{UV } \lambda_{\text{max}} (\log \epsilon): \ 237 (4.15), \ 285 (3.88), \ 358 (4.10) \text{ nm}.
\]

\[
^1\text{H-NMR} \ (400 \text{ MHz, CDCl}_3) \delta: \ 2.68 \ (1 \text{ H, dd, } H(7'), J = 14.5, 11.0 \text{ Hz}), \ 3.06 \ (1 \text{ H, dd, } H(7'), J = 14.5, 4.6 \text{ Hz}), \ 3.81-3.90 \ (1 \text{ H, m, } H(3)), \ 3.97 \ (6 \text{ H, s, } -\text{OC}_6\text{H}_5), \ 4.27 \ (1 \text{ H, dd, } H(4), J = 9.5, 3.0 \text{ Hz}), \ 4.31 \ (1 \text{ H, dd, } H(4), J = 9.5, 6.5 \text{ Hz}), \ 5.82 \ (1 \text{ H, s, } -\text{OH}, \text{ disappears upon addition of } D_2O), \ 5.94 \ (2 \text{ H, m, } -\text{OC}_6\text{H}_5), \ 6.62 \ (1 \text{ H, d, } H(2'), J = 1.5 \text{ Hz}), \ 6.65 \ (1 \text{ H, dd, } H(6'), J = 7.5, 1.5 \text{ Hz}), \ 6.74 \ (1 \text{ H, d, } H(5'), J = 7.5 \text{ Hz}), \ 6.83 \ (2 \text{ H, s, } H(2'')), \ 7.58 \ (1 \text{ H, d, } H(7''), J = 2 \text{ Hz}). \text{ Irradiation of } H(7')(\delta 3.06) \text{ simplified } H(3) \text{ multiplet and collapsed}
\]
H(7')(δ2.68) to a doublet. Irradiation of both H(4) protons also simplified the H(3) multiplet.

$^{13}$C-NMR (75.3 MHz, CDCl$_3$) δ: 37.539 (C(7)), 39.489(-) (C(3)), 56.402(-) (-OCH$_3$), 69.618 (C(4)), 101.085 (-OCH$_2$O-), 107.172(-) (C(2")), 108.428(-), 108.957(-), 121.796(-), 125.355, 125.491, 131.335, 136.901, 137.928(-) (C(7"), 147.169, 147.946, 172.562 (C(1)).

El MS m/z: 384 (M$^+$), 249, 135. Exact mass calculated for C$_{21}$H$_{20}$O$_7$: 384.1209; found: 384.1219.

4.21. (±)-5,5*-BIS(1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-6-HYDROXY-3-HYDROXYMETHYL-7-METHOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID γ LACTONE) (83) AND (±)-1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-8-HYDROXY-3-HYDROXYMETHYL-7-METHOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID γ LACTONE (84)
C. roseus CFE (pH 6.3, specific activity 1.5 units/mg, 215 mL, 330 units) was added to a solution containing butanolide 58 (1.860 g, 4.79 mmol) in ethanol (25 mL), water (100 mL) and hydrogen peroxide (20 mL 0.17 M solution, 3.4 mmol). After stirring for 1 h, further hydrogen peroxide (10 mL, 1.7 mmol) was added and stirring continued for an additional 4.5 h. The solution was extracted by the standard procedure and the dichloromethane extract purified by column chromatography using acetone/dichloromethane (1:4 to 1:0, v/v) to afford aryletralin 59 (901 mg, 48%), dimer 83 (680 mg, 37%) and aryltetralin 84 (9.8 mg, 0.53%).

Physical properties of 83

Mp 156-168° C

IR νmax (CHCl3): 3540, 1780 cm⁻¹.

1H-NMR (300 MHz, acetone-d6) δ: 2.40-2.52 (3 H, m, H(2)(twice), H(4)), 2.55-2.68 (2 H, m, H(3)), 2.90 (1 H, br dd, H(4), J = 14, 13 Hz), 2.98 (1 H, dd, H(4), J = 14, 4 Hz), 3.48-3.55 (1 H, m, H(4)), 3.59 (3 H, s, -OCH₃(7)), 3.67, 3.67 (3 H total, s, s, -OCH₃(7)), 3.75 (6 H, s, -OCH₃(3')), 3.83, 3.83 (6 H total, s, s, -OCH₃(3')), 3.95-4.03 (2 H, m, H(11)), 4.14 (2 H, br d, H(1), J = 12 Hz), 4.48-4.56 (2 H, m, H(11)), 5.44, 5.55, 6.02, 6.05 (4 H total, s, s, s, -OH), 6.18 (1 H, s, -OH(6)), 6.36, 6.36 (1 H total, s, s, H(8)), 6.45 (2...
Physical properties of 84

\( ^1 \)H-NMR (400 MHz, CDCl\(_3\)) \( \delta: \) 2.36 (1 H, m, H(2)), 2.59-2.74 (2 H, m, H(3), H(4)), 3.08 (1 H, dd, H(4), \( J = 16, 2 \) Hz), 3.60 (3 H, s, -O\( \text{CH}_3 \)), 3.74 (1 H, d, H(1), \( J = 11 \) Hz), 3.80, 3.89 (3 H each, s, s, -O\( \text{CH}_3 \)), 4.09 (1 H, dd, H(11), \( J = 10, 9 \) Hz), 4.61 (1 H, dd, H(11), \( J = 10, 7 \) Hz), 5.38, 5.43 (1 H each, s, s, -OH(6), -OH(4')), 5.56, 6.09 (1 H each, d, d, H(2'), H(6'), \( J = 2.5 \) Hz each), 6.77, 6.85 (1 H each, d, d, H(5), H(6), \( J = 8 \) Hz each).

El MS m/z: 386 (M\(^+\)), 154, 139. Exact mass calculated for \( C_{21}H_{22}O_7 \): 386.1366; found: 386.1373.

4.22. ATTEMPTED BIOTRANSFORMATION OF TRANS-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL)BUTANOLIDE (58) WITHOUT HYDROGEN PEROXIDE

\( C. \text{roseus} \) CFE (pH 6.3, specific activity 2.5 units/mg, 30.4 mL, 47 units) was added to a solution containing butanolide 58 (61.0 mg, 2.32 mmol), ethanol (3.8 mL), potassium phosphate buffer (0.100 M, pH 6.3, 43.0 mL), water (20.2 mL). The solution was stirred for 3 h and extracted by the standard procedure using only dichloromethane to afford butanolide 58 (59.3 mg, 97% recovery) which was pure by \( ^1 \)H-NMR spectroscopy.
4.23. ATTEMPTED DIMERIZATION OF 1-(3,5-DIMETHOXY-4-HYDROXYPHENYL)-6-HYDROXY-3-HYDROXYMETHYL-7-METHOXY-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID γ LACTONE (59) WITHOUT CFE

Aryltetralin 59 (15.2 mg, 39.3 μmol) was dissolved in a solution of ethanol (7.3 mL), water (6.7 mL), hydrogen peroxide (2.16 mL 81.6 mM solution, 1.76 mmol) and potassium phosphate buffer (0.100 M, pH 6.3, 8.9 mL). The solution was stirred for 17 h and analyzed by HPLC: no products were detected.

4.24. (±)-2-HYDROXY-8-HYDROXYMETHYL-6,7,8,9-TETRAHYDRO-3,3',5'-TRI-METHOXYSPIRO[5H-BENZOCYCLOHEPTENE-5,1'-CYCLOHEXA-2',5'-DIEN]-4'-ONE-7-OIC ACID γ LACTONE (85)(LARGE SCALE EXPERIMENT, SECTION 2.4.)

\[ HO \quad 2 \quad 1 \quad 9 \quad 8 \quad 7 \quad 6 \quad 5 \quad 4 \quad 3 \quad 4' \quad O \quad CH_3 \]

\[ CH_3O \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8 \quad 9 \quad 1 \quad 2 \quad OCH_3 \]

C. roseus CFE (pH 6.3, specific activity 2.5 units/mg, 450 mL, 690 units) was added to a solution containing butanolide 58 (900 mg, 2.32 mmol), ethanol (90 mL), water (150 mL) and hydrogen peroxide (30 mL 0.17 M solution, 5.1 mmol). The solution was stirred for 3 h and extracted by the standard procedure. The dichloromethane extract was purified by column chromatography using ethyl acetate/chloroform (1:4 to 9:11, v/v) to afford aryltetralin 59 (633 mg, 71%), butanolide 58 (56 mg, 6%) and spirodienone 85 (9.7 mg, 1.1%).
Physical properties of 85

Mp 225-230° C

IR $\nu_{\text{max}}$ (CHCl$_3$): 3539, 1774, 1673 cm$^{-1}$.

UV $\lambda_{\text{max}}$ (log e): 272 (3.83) nm.

$^1$H-NMR (400 MHz, CDCl$_3$) $\delta$: 2.03 (1 H, dd, H(6), $J = 13.3, 12.8$ Hz), 2.32 (1 H, dd, H(6), $J = 13.7, 2.6$), 2.32-2.44 (1 H, m, H(8)), 2.68 (1 H, ddd, H(7), $J = 12.6, 12.6, 2.6$ Hz), 2.97 (1 H, dd, H(9), $J = 15, 2.3$ Hz), 3.07 (1 H, dd, H(9), $J = 14, 10.9$ Hz), 3.65 (3 H, s, -OCH$_3$(3)), 3.79, 3.84 (3 H each, s, s, -OCH$_3$(3'), -OCH$_3$(5')), 3.98 (1 H, dd, -CH$_2$OCO-, $J = 9, 8.4$ Hz), 4.51 (1 H, dd, -CH$_2$OCO-, $J = 9, 7.6$ Hz), 5.55 (1 H, br s, -OH(2), disappears upon addition of D$_2$O), 6.13, 6.25 (1 H each, d, d, H(2), H(6'), $J = 2.8$ Hz each), 6.78, 6.92 (1 H each, s, s, H(1), H(4)).

Irradiation of H(7) collapses H(6) signals to doublets ($J = 13.6$ Hz each) and simplifies H(8) multiplet.

EI MS m/z: 386 (M$^+$). Exact mass calculated for C$_{21}$H$_{22}$O$_7$: 386.1366; found: 386.1362.

4.25. BIOTRANSFORMATION OF (±)-TRANS-2-(3,5-DIMETHOXY-4-
HYDROXYBENZYL)-3-(3,4-DIHYDROXY-α-HYDROXYBENZYL)BUTANOLIDE (94)

4.25.1. REACTION AND EXTRACTION

Hydrogen peroxide (4.58 mL 81.6 mM solution, 0.37 mmol) and C. roseus CFE (pH 7.0, specific activity 0.51 units/mg, 22.1 mL, 150 units) were added in rapid succession to a solution containing butanolide 94 (73.2 mg, 0.187 mmol), ethanol (4.6 mL), water (14.5 mL) and phosphate buffer (0.100 M, pH 7.00, 63.7 mL). The solution turned dark purple within 1 min. After 10 min of stirring, ethyl acetate (80 mL) and Celite were added and the suspension was stirred vigorously for an additional 5 min and then filtered. Additional ethyl acetate (320 mL) was added and the phases were separated. The organic extract was dried over magnesium sulfate, filtered and evaporated in vacuo to yield a brown solid (60.3 mg). The aqueous residue was subjected to a continuous extraction with ethyl acetate for 2 d. The organic extract was dried over magnesium sulfate, filtered and evaporated in vacuo to afford a brown
solid (7.6 mg) which was combined with the previous ethyl acetate extract. The aqueous residue was evaporated \textit{in vacuo} and stirred \textit{in refluxing} methanol (100 mL) for 16 h. The suspension was filtered while hot and evaporated \textit{in vacuo} to yield brown solids (205.1 mg).

4.25.2. CONTROL EXPERIMENT

Butanolide 94 (4.20 mg, 10.8 μmol) was dissolved in a solution of ethanol (0.28 mL), water (0.87 mL), and potassium phosphate buffer (0.100 M, pH 7.0, 5.16 mL). Hydrogen peroxide (0.28 mL 81.6 mM solution, 23 μmol) was added and the solution was stirred for 10 min prior to being extracted with ethyl acetate (2 x 50 mL). The organic extract was dried over magnesium sulfate, filtered and evaporated \textit{in vacuo}. The product (4.01 mg, 95% recovery) was found to be pure butanolide 94 by $^1$H-NMR spectroscopy and TLC.

4.25.3. ETHYL ACETATE EXTRACT

4.25.3.1. REDUCTION: (±)-4-(3,4-DIHYDROXYPHENYL)-5,7-DIMETHOXY-6-HYDROXY-2-HYDROXYMETHYL-1,2,3,4-TETRAHYDRO-2-NAPHTHOIC ACID γ LACTONE (103)

A sample of the ethyl acetate extract (15.47 mg) was dissolved in a mixture of methanol (5 mL) and water (1 mL). Sodium borohydride (1.6 mg, 42 μmol) was added, resulting in an instantaneous color change of the solution from reddish-brown to yellow. After stirring for 5 min, 2 M hydrochloric acid (2 drops) was added, followed by water (2 mL) and brine (1 mL). The mixture was extracted with ethyl acetate (30 mL) and the organic extract was dried over magnesium sulfate, filtered and evaporated \textit{in vacuo} to afford a brown oil (16.62 mg). Purification by preparative TLC using acetone/dichloromethane (2:3, v/v) gave the aryltetralin 103 (2.23 mg, 14.0%) and 94 (2.06 mg, 12.3%).
4.25.3.2. METHYLATION: (±)-2-(3,5-DIMETHOXY-4-HYDOXYBENZYL)-3-(3-HYDROXY-4-METHOXYBENZYL-α-HYDROXY)BUTANOLIDE (114a) AND (±)-2-(3,5-DIMETHOXY-4-HYDROXYBENZYL)-3-(4-HYDROXY-3-METHOXYBENZYL-α-HYDROXY)BUTANOLIDE (114b)

Diazomethane in ethereal solution (approximately 0.4 M) was added to a sample of the ethyl acetate extract (23.19 mg) dissolved in dry methanol (5 mL) until no further effervescence was observed. After stirring for 22 h, aqueous acetic acid (10%, 2 drops) was added and the solution evaporated in vacuo. The residue was purified by preparative TLC using acetone/dichloromethane (1:4, v/v) to yield 94 (3.66 mg, 15%) and 114 (2.38 mg, 9%, 1:1 mixture of isomers by $^1$H-NMR spectroscopy).

Physical properties of 114 mixture

IR $\tilde{\nu}_{max}$ (CHCl$_3$): 3600, 3540, 1770 cm$^{-1}$.

$^1$H-NMR (300 MHz, acetone-d$_6$ + D$_2$O) δ: 2.64-2.85, 2.85-3.02 (8 H total, m, m, H(2), H(3), H(7')), 4.02-4.15 (4 H, m, H(4)), 4.72 (1 H, d, H(7'), J = 5.1 Hz), 4.76 (1 H, d, H(7'), J = 5.1 Hz), 6.39 (2 H, s, H(2')), 6.40 (2 H, s, H(2')), 6.70-6.92 (6 H, m, H(2'), H(5'), H(6')).

EI MS m/z: 418 (M$^+$), 400, 181, 167, 153. Exact mass calculated for C$_{22}$H$_{26}$O$_8$: 418.1628; found: 418.1633.
4.25.3.3. REDUCTION AND METHYLATION: (+)-4-(3,4-DIMETHOXYPHENYL)-3-
HYDROXYMETHYL-1,2,3,4-TETRAHYDRO-5,6,7-TRIMETHOXY-2-NAPHTHOIC ACID γ
LACTONE (115) AND UNIDENTIFIED COMPOUND 116

Sodium borohydride (42 mg, 1.1 mmol) was added to a sample of the ethyl acetate extract (26.94 mg) dissolved in a mixture of methanol (4.5 mL) and water (0.5 mL) and the bright yellow solution was stirred at room temperature for 1 h. The solution was acidified with 1 M hydrochloric acid and water (5 mL) and brine (2 mL) were added. The solution was extracted with ethyl acetate (2 x 60 mL) and the extract was dried over magnesium sulfate, filtered and evaporated in vacuo before being re-dissolved in ethanol (5 mL). Freshly-prepared ethereal diazomethane (approximately 0.3 g, 7 mmol) was added and the solution stirred for 15 h. Aqueous acetic acid (10%, 0.2 mL) was added and the solution evaporated in vacuo to afford a yellow oil (67.5 mg) which was separated by column chromatography using acetone/dichloromethane (1:19 to 1:0 gradient, v/v) to afford 42.92 mg (64%) of eluted products, including 116 (8.56 mg, 25%) and 115 (6.00 mg, 20%). The column packing was removed and stirred in a mixture of acetone (50 mL), water (30 mL) and calcium carbonate (700 mg) at 45°C for 1 h. The mixture was acidified with 2 M hydrochloric acid and the solids filtered and washed with warm acetone (2 x 30 mL). The filtrate was evaporated in vacuo and the final traces of silica removed to afford a yellow oil (12.27 mg, no identifiable material by 1H-NMR spectroscopy) for a total column recovery of 82%.
Physical properties of 115

IR $\nu_{\text{max}}$ (CHCl$_3$): 1770 cm$^{-1}$.

$^1$H-NMR (200 MHz, acetone-d$_6$) $\delta$: 2.29-2.74 (2 H, m, H(2), H(3)), 2.97-3.09 (1 H, m), 3.14 (3 H, s, -$\text{OCH}_3$(5)), 3.37 (1 H, dd, $J = 7.0$, 6.6 Hz), 3.5-3.6 (1 H, m), 3.69-3.91 (12 H, m, -$\text{OCH}_3$), 4.04-4.18 (1 H, m), 4.22 (1 H, br d, H(4), $J = 8.0$ Hz), 6.60 (1 H, m, H(6')), 6.72-6.84 (2 H, m, H(2'), H(8)), 6.90 (1 H, d, H(5'), $J = 8$ Hz).

El MS m/z: 414 (M$^+$), 400, 181, 151, 137. Exact mass calculated for C$_{23}$H$_{26}$O$_7$: 414.1679; found: 414.1678.

Physical properties of 116

IR $\nu_{\text{max}}$ (CHCl$_3$): 3320, 2970, 2940, 2870, 1670 cm$^{-1}$.

El MS m/z: 432 (M$^+$-H$_2$O?), 418, 282, 167.

4.25.4. METHANOL EXTRACT

4.25.4.1. METHYLATION

Diazomethane in ethereal solution (approximately 0.4 M) was added to a sample of the methanol extract (90.82 mg) dissolved in dry methanol (10 mL) until no further effervescence was observed. The yellow solution was stirred overnight. Analysis by silica TLC showed no new spots in comparison with the initial methanol extract. In both cases no spots with an $R_f$ value greater than zero were observed using acetone/dichloromethane (1:4, v/v).
4.25.4.2. REDUCTION AND METHYLATION

Sodium borohydride (137 mg, 3.6 mmol) was added to a sample of the methanol extract (108.65 mg) dissolved in a mixture of methanol (9 mL) and water (1 mL) and the solution was stirred at room temperature for 1 h. The solution was acidified with 1 M hydrochloric acid and water (5 mL) and brine (2 mL) were added. The solution was extracted with ethyl acetate (3 x 60 mL) and the extract was dried over magnesium sulfate, filtered and evaporated in vacuo before being re-dissolved in dry THF (10 mL). Potassium carbonate (180 mg, 1.3 mmol) and methyl iodide (0.5 mL, 8 mmol) were added and the solution stirred for 1 h. Water (2 mL) was added and the mixture acidified with 2 M hydrochloric acid before being extracted with ethyl acetate (2 x 20 mL). The organic extract was dried over magnesium sulfate, filtered and evaporated in vacuo to afford brown solids (43.9 mg). Sonication in dichloromethane followed by filtration and evaporation in vacuo gave tan solids (5.35 mg) which exhibited no aromatic proton signals by $^1$H-NMR spectroscopy.
Fig. 3a) 200 MHz $^1$H-NMR Spectrum of Butanolide 58; b) Expansion of Aromatic Region.
Fig. 4. 200 MHz $^1$H COSY NMR Spectrum of Butanolide 58.
Fig. 5. El Mass Spectrum of Butanolide 58.
Fig. 6. El Fragmentation Pattern of Butanolide 58.
Fig. 7. 200 MHz $^1$H-NMR Spectrum of Aryltetralin 59.
Fig. 8. 200 MHz $^1$H COSY NMR Spectrum of Aryltetralin 59.
Fig. 9. El Mass Spectrum of Aryltetralin 59.
Fig. 10. EI Fragmentation Pattern of Aryltetralin 59.
Fig. 11. 300 MHz $^1$H-NMR Spectrum of Butanolide 76 (Aromatic Region).
Fig. 12. El Mass Spectrum of Butanolide 76.
Fig. 13. El Fragmentation Pattern of Butanolide 76.
Fig. 14. El Mass Spectrum of Arylteralin Dimer B3.
Fig. 15. 300 MHz $^1$H-NMR Spectrum of Spirodienone 85.
Fig. 16. El Mass Spectrum of Spirodienone 85.
Fig. 17. 200 MHz $^1$H-NMR Spectrum of Dithioketal 96.
Fig. 18. 200 MHz $^1$H COSY NMR Spectrum of Dithioketal 96.
Fig. 19. El Mass Spectrum of Dithioketal 96.
Fig. 20. 200 MHz $^1$H-NMR Spectrum of Butanolide 94.
Fig. 21. El Mass Spectrum of Butanolide 94.
Fig. 22. 200 MHz $^1$H-NMR Spectrum of Aryltetralin 103.
Fig. 23. El Mass Spectrum of Aryltetralin 103.
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