

INVESTIGATIONS INTO THE ASYMMETRIC  
SYNTHESIS AND BIOLOGICAL ACTIVITIES OF SOME  
BISBENZYL TETRAHYDROISOQUINOLINE ALKALOIDS

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**INVESTIGATIONS INTO THE  
ASYMMETRIC SYNTHESIS AND BIOLOGICAL ACTIVITIES  
OF SOME BISBENZYL TETRAHYDROISOQUINOLINE ALKALOIDS**

by

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(B.Sc. Acadia University, 1999)

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## ABSTRACT

Bisbenzyltetrahydroisoquinoline (BBIQ) alkaloids are a very large and structurally diverse family of compounds that have been isolated from a variety of plant sources and have been found to exhibit a multitude of pharmacological properties including antitumor, antimalarial, and antibacterial activities. Variations in the number of aromatic oxygen substituents present, the number of ether linkages, the nature of the ether bridges and the sites on the two benzylisoquinoline units where the ether or carbon-carbon bond originate, have made BBIQs very interesting targets for several synthetic endeavours.

The research described herein will focus on the anti-inflammatory and antioxidant activities of the BBIQ oxyacanthine, and several other compounds isolated from the herbal plant *Mahonia aquifolium*, using the lipoxygenase inhibition and DPPH radical scavenging assays. In addition, the total synthesis of two target BBIQ are investigated utilizing the Pictet-Spengler and Bischler-Napieralski strategies for isoquinoline formation. Furthermore, alternative means to the classic Ullmann conditions for ether linkages is explored.

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### List of Abbreviations

AcOH	acetic acid
Bn	benzyl
BOC	<i>tert</i> -butoxycarbonyl
BNC	Bischler-Napieralski cyclization
C.A.	chiral auxillary
CBZ <sub>o</sub>	carbobenzoxyl
COSY	homonuclear correlated spectroscopy
CH <sub>2</sub> Cl <sub>2</sub>	dichloromethane
d	day(s)
DCC	dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DME	ethylene glycol dimethyl ether
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOAc	ethyl acetate
EtOH	ethyl alcohol
FCC	flash column chromatography
h	hour(s)
HMBC	heteronuclear correlation through multiple bond coherence

HRMS	high resolution mass spectrum
IL	interleukin
<i>i</i> -Pr	isopropyl
LC-MS	liquid chromatography-mass spectroscopy
min	minute(s)
Me	methyl
MeOH	methyl alcohol
MES	morpholinoethanesulfonic acid
<i>n</i> -BuLi	<i>n</i> -butyllithium
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
PAF	platelet activating factor
PBS	phosphate buffered saline
PCC	pyridinium chlorochromate
PG	protecting group
Ph	phenyl
PLC	preparative layer chromatography
<i>p</i> -TSA	<i>para</i> -toluenesulfonic acid
rt	room temperature
s	second(s)
TBDMS	<i>tert</i> -butyldimethylsilyl

THF

tetrahydrofuran

TMEDA

*N,N*-tetramethylethylenediamine

TNF

tumor necrosis factor

## CHAPTER I

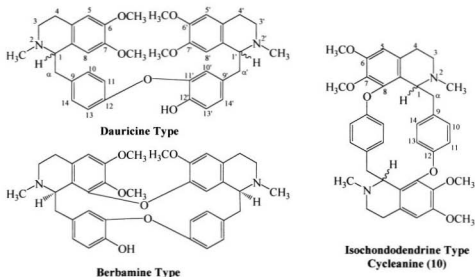
### INTRODUCTION

#### 1.1 Structures and Sources of BBIQ Alkaloids

Bisbenzyltetrahydroisoquinoline (BBIQ) alkaloids, somewhat erroneously referred to in many publications as “bisbenzylisoquinolines” are almost forgotten targets in synthetic chemistry ever since they were first identified and studied in the mid-1970's. With the rapid discovery of other novel, complex compounds providing intriguing pharmacological promise and synthetic challenges, BBIQs took a back seat with respect to synthetic interest. On the other hand, biologists, biochemists, and immunologists have continued to investigate these compounds for the variety of pharmacological properties that they exhibit.

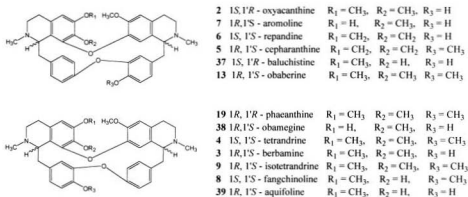
The BBIQ structural class of alkaloid is very large with a modest estimate of over 200 members.<sup>1,2</sup> They have been isolated from a variety of plant sources, with their amounts and structures being determined by ecological factors.<sup>3</sup> Their molecular structures vary according to the number of aromatic oxygen substituents and the number and nature of the ether bridges, as well as the sites on the BBIQ units at which the ether or carbon-carbon bonds originate.<sup>3</sup> Considering the extremely large number of possible variations, the BBIQs are divided into 28 categories according to their structural compliment. Figure 1 represents three different structures: (a) the dauricine type, characterized by a single ether bridge between the two benzyl groups; (b) the berbamine type, with ether bridges between the two benzyl groups and also between the two





**Figure 1.** Three structural “types” of BBIQ alkaloids.

tetrahydroisoquinoline units, with the substitution pattern shown; and (c) the isochondodendrine type which can be considered to be “head-to-tail” dimers. An extensive database has been compiled which categorize these compounds according to their botanical sources.<sup>1</sup> Many of these sources exhibit potent pharmacological



activities,<sup>4,5,6,7,8</sup> which have been in part attributed to the action of BBIQs.

*Mahonia aquifolium* is an ornamental shrub of particular interest. Its extracts have been reported to include potent antioxidant, antiproliferative, and anti-inflammatory effects. The investigations of Müller *et al.*<sup>5</sup> and Mišik *et al.*<sup>4</sup> have provided evidence that some of the constituent alkaloids of this plant exhibit new potent, non-toxic treatments for psoriasis and other inflammatory conditions. Their work, as well as the investigations of this author will be examined in the following chapters.

## 1.2 Pharmacological Activity of BBIQ Alkaloids

The first pharmacological interest in these alkaloids stems from the known use of one of these compounds as a poison in arrows used by several native South American tribes. (+)-Tubocurarine (**1**), which is isolated from a South American plant, serves to

block the action of acetylcholine, resulting in muscle paralysis and eventually leading to death through respiratory failure. The compound was later utilized in conjunction with other anesthetics in abdominal surgery as a muscle relaxant.<sup>2</sup> Since this initial interest, much attention has been focussed on determining additional pharmacological activities of BBIQs, only some of which are outlined in Table 1.

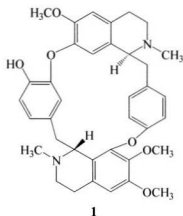


Table 1 reveals that anti-inflammatory action is common to each of the compounds and that there is a multitude of activities which have been observed for the oxyacanthine (**2**)-

**Table 1.** Some pharmacological activities attributed to the action of BBIQs.

Compound	Structure	Pharmacological Effects
oxyacanthine	2	anti-inflammatory, <sup>9,10</sup> antioxidant, <sup>6</sup> antiproliferative, <sup>10</sup> inhibition of IL-2 and TNF <sup>11</sup>
berbamine	3	antiplasmodial, <sup>12</sup> anti-inflammatory, <sup>9,10,13</sup> antiproliferative, <sup>10</sup> antioxidant, <sup>6</sup> inhibition of PAF, <sup>14,15</sup> inhibition of IL-2 and TNF, <sup>11</sup> immunosuppressive <sup>15</sup>
tetrandrine	4	Inhibition of T-cell dependant immune responses, <sup>8</sup> antiplasmodial, <sup>12</sup> anti-inflammatory, <sup>10,13,16,14</sup> PAF inhibition <sup>14</sup>
cepharanthine	5	anti-inflammatory, <sup>11</sup> antiperoxidation, suppression of potassium release from red blood cells, inhibition of erythrocyte sick cell formation, platelet aggregation <sup>17</sup>
(-)-repaandine	6	antiplasmodial, <sup>12</sup> anti-inflammatory <sup>4,11</sup>
aromoline	7	" "
fangchinoline	8	" "
isotetrandrine	9	" "
cycleanine	10	" "

berbamine (**3**) types of structures. These two compounds are of particular interest to the research presented in this thesis, firstly because **2** and **3** were isolated from *Mahonia aquifolium*, and secondly because the structures are very similar, except for differences in their substitution patterns and opposite configurations at C1 and C1'. In addition, the structures of many other BBIQs (i.e., **4-9**) are variants of the oxyacanthine-berbamine type, therefore studies into pharmacological effects and synthesis may be significant for other compounds.

Inflammation and oxidation have pronounced negative effects in biological systems. BBIQs are potentially potent, non-toxic therapeutic agents which have been demonstrated to inhibit these activities. The research conducted by this author, and is described in this thesis, will examine the abilities of the oxyacanthine-berbamine type of compounds to inhibit the action of inflammation and oxidation. This is accomplished using *in vitro* lipoxigenase inhibition and DPPH radical scavenging assays, techniques which will be described in detail in Chapters 2 and 3, respectively.

Over the past 30 years, several attempts have been made to determine the structure-activity correlations for BBIQs.<sup>2,18</sup> Some investigations lend support to chirality and substitution patterns aiding in the selective interaction with various substrates,<sup>11,12</sup> while others suggest that the BBIQ is metabolized to active monomer units. Angehofer *et al.*<sup>12</sup> recently reported Selectivity Indices (SI) for 53 BBIQs, based on measuring the ratios of their antiplasmodial activity to their cytotoxicity. They were able to identify some trends in compounds that exhibited high SI values, however no conclusions could be made for the other alkaloids tested. Although investigations have provided evidence for many different hypotheses, definitive proof of how these compounds exert their effects in biological systems has still not been provided. What is known is that BBIQs do exhibit potent biological activities and represent a potentially non-toxic alternative to many commercial therapeutic agents.

### **1.3 Synthesis of BBIQs**

Few synthetic initiatives have been undertaken since the 1960's and 70's. The

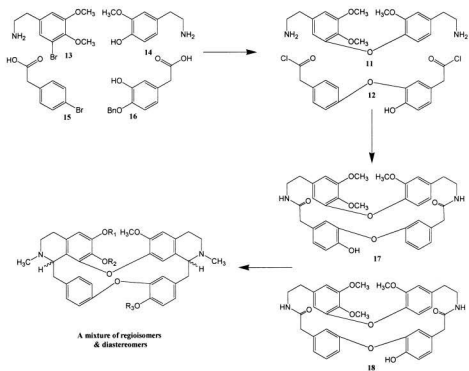
attempts during this period are outlined in a couple of reviews by Shamma *et al.*,<sup>2,18</sup> with particular emphasis on the work conducted by Tomita,<sup>18,19,22</sup> Inubushi<sup>23</sup> and Kametani.<sup>24-26</sup> The latter two researchers focused primarily on the synthesis of oxyacanthine (**2**)-berbamine (**3**) and related structures, while Tomita concentrated on the synthesis of the isochondrodendrine type of BBIQ (*e.g.* cycleanine, **10**). Each group undertook similar synthetic routes to their respective compounds, thus encountering many of the same problems associated with regioselectivity, diastereoselectivity and coupling difficulties.

#### **1.4 Previous Synthetic Approaches Towards Oxyacanthine and Berbamine**

The synthetic endeavours of the groups of Inubushi and of Kametani had several features in common, including:

- 1) derivation from four similarly substituted synthons,
- 2) ether linkages formed via some variation of the classical Ullmann conditions,
- 3) amides formed either by the Schotten-Baumann reaction or a DCC-aided condensation between a phenylacetic acid and an amine, and
- 4) tetrahydroisoquinoline formation accomplished via a Bischler-Napieralski cyclization.

Scheme 1 shows the synthetic approach proposed by Kametani *et al.*, in which the BBIQ macrocycle was formed from the combination of “top” (**11**) and “bottom” (**12**) halves. Intermediates **11** and **12** were formed in low yield (10%) via ether linkage of the two amine synthons (**13** & **14**) and the two acid synthons (**15** & **16**) respectively. Following the construction of the bisamide regioisomers (**17** & **18**), Bischler-Napieralski cyclization

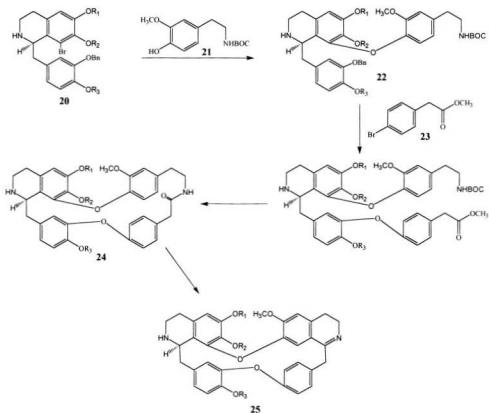


**Scheme 1**

and  $\text{NaBH}_4$  reduction successfully formed the two tetrahydroisoquinoline rings and resulted in the formation of the two stereogenic centers at C1 and C1' as a mixture of diastereomers that proved to be very difficult to separate.

Inubushi *et al.* proposed the route shown in Scheme 2 to compounds **9**, **19** and **4**. They originally considered that two appropriately substituted benzyltetrahydroisoquinoline units could be joined via a dual Ullmann coupling to give the macrocycle. However, this approach would give rise not only to the oxyacanthine-berbamine (head-to-head, tail-to-tail coupling) type of structure, but also to an isochondodendrine (head-to-

tail, *e.g.* cycleanine (**10**)) type of structure. Inubushi circumvented this problem through synthesis of the bromine-substituted benzyltetrahydroisoquinoline (**20**), thus allowing a stepwise route that permitted selective formation of ether linkages and of tetrahydroisoquinoline cyclizations (Scheme 2). They reported the resolution of *R*-(-)-*O*-benzyl-8-bromolaudanidine (**20**) as its (+)-tartaric salt, followed by an Ullmann reaction with **21** to give **22**. Deprotection of the phenol by hydrogenation and a second Ullmann reaction



**Scheme 2**

condensation with **23**, formed the ether linkage for the bottom half of the structure. At this juncture, removal of the *t*-butoxycarbonyl group and saponification of the ester allowed for condensation with the amine to give amide **24**. Fortunately, the positioning of the electron-donating groups on the aromatic ring permitted the formation of only one regioisomer (**25**).

In general, Inubushi's route (Scheme 2) was the most effective, circumventing many of the problems in regioselectivity and diastereoselectivity in Kametani's method (Scheme 1). However, both attempts still had difficulties associated with the Ullmann reaction as well as controlling the stereochemical outcome of their final products.

### 1.5 Cycleanine

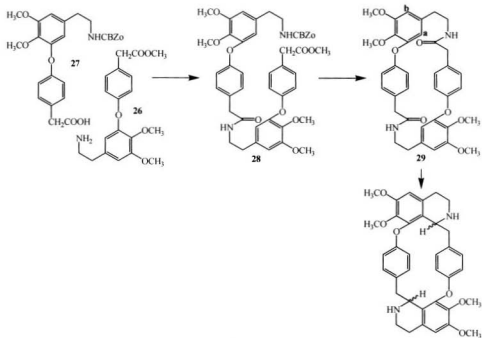
Cycleanine (**10**) is a symmetrical BBIQ that is an interesting synthetic and medicinal target. The structure of **10** is clearly a head-to-tail dimer composed of two benzyltetrahydroisoquinoline monomer units. Considering that the functional groups present are limited compared to other, more complex BBIQs, the compound may be an ideal candidate for studies on structure-activity correlations. As well, interest originating from the DPPH radical scavenging assay, permits an examination of the role of tetrahydroisoquinolines as radical reducing compounds. From these perspectives, the synthesis of cycleanine (**10**) would serve as a model for examining the effectiveness of modern techniques and reagents for the synthesis of the more complex BBIQs.

### 1.6 Synthetic Approaches to Cycleanine

Previous attempts towards the synthesis of the isochondodendrine type of BBIQ.



cycleanine (**10**) can be accredited to the work of Tomita *et al.*<sup>19-22</sup> The fact that cycleanine is a head-to-tail dimer permits exploitation of symmetry in the total synthesis. Tomita proposed two strategies. The first is outlined in Scheme 3. It is an approach to the construction of a bis-amide followed by a subsequent dual Bischler-Napieralski reaction to form the two tetrahydroisoquinoline units. The synthetic sequence involved the initial DCC-aided condensation between the unprotected amine group of compound **26** and the

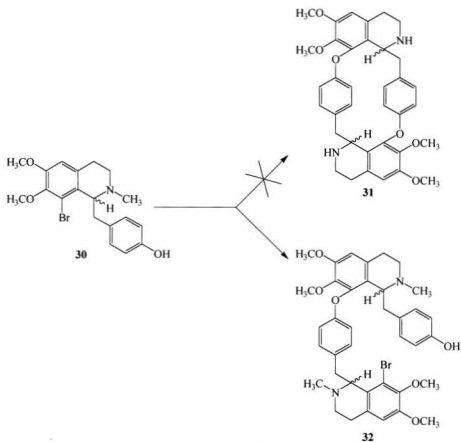


**Scheme 3**

phenylacetic acid functionality of compound **27**. The resultant CBZ-protected amine **28** was deprotected, and then saponification of the phenylacetic ester and a second DCC

aided condensation gave bis-amide **29**. As in Kametani's approach to oxyacanthine-berbamine, the Bischler-Napieralski cyclization resulted in a mixture of regioisomers (ring closure on both sites **a** and **b**). Since formation of neither of the tetrahydroisoquinoline regioisomers could be controlled, the reaction resulted in a complex mixture of products.

Tomita recognized the problems associated with this approach and developed the



**Scheme 4**

strategy illustrated in Scheme 4. Tomita attempted to make use of the symmetrical nature of cycleanine and construct an appropriately substituted monomer unit which he hoped to dimerize via a dual Ullmann ether linkage reaction. The synthesis of *dl*-bromo-armepavine (**30**) was very efficient, resulting in the correct regioisomer as the predominant product. However, the second step involving the dual Ullmann ether couplings did not give the desired product (**31**). Instead, formation of only one of the ether linkages was effected (**32**), but the second ether linkage was not successful despite further attempts.

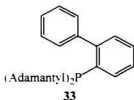
A great deal of effort has been invested into the synthesis of the oxyacanthine-berbamine and cycleanine structures, however each route, regardless of the structure type, incurred problems with the formation of the macrocyclic ether linkages and with determining the correct regiochemical outcome of the tetrahydroisoquinoline ring closure. Unfortunately, researchers were greatly limited with respect to alternatives for these key steps. Nonetheless, with the development of modern methodologies, the potential for overcoming these difficulties may be greatly enhanced.

### **1.7 Modern Alternatives to the Ullmann Reaction**

The original Ullmann conditions involved the use of elevated reaction temperatures, formidable purification problems, generally low yields and the use of stoichiometric amounts of copper.<sup>27</sup> In addition, the coupling was only effective for compounds that contained inactivated or slightly activated halides,<sup>28</sup> a condition which posed significant difficulties for BBIQ structures containing highly activated and



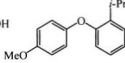


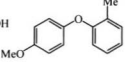
sterically hindered halides. Fortunately, as a result of the discovery of large, highly substituted macrocyclic species such as vancomycin, the synthetic organic community has seen the rise of many alternative methods for constructing diaryl ether linkages. Evans<sup>59</sup> and Chan<sup>60</sup> reported the use of arylboronic acids to couple with activated phenols through the mediation of  $\text{Cu}(\text{OAc})_2$ . Although the reaction has been reported to tolerate a wide variety of substituents on both coupling partners, in cases where the aryl halide has an *ortho*-heteroatom substituent, the yields of product are generally low. However, reactions between halides and highly activated phenols have enjoyed much success and have been reported in yields that are substantially higher than those produced under traditional Ullmann conditions.

In addition, Buchwald *et al.*<sup>11</sup> reported on diaryl-ether linkages of deactivated, neutral, and highly activated aryl halides utilizing a palladium-catalysed reaction with several different bases, solvents and biphenylphosphine ligands, depending on the type of system presented. For highly activated aryl halides substituted with electron-donating groups in the *para* position, high yields were reported using the biphenyl ligand **33** and a monobasic potassium phosphate base (Table 2). However,



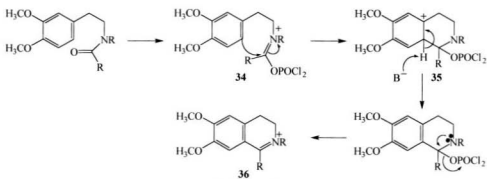
they provided no example of a successful coupling reaction of electron-donating groups *ortho* to the halide. Also, no explanation was provided as to why such a sterically bulky ligand was necessary to effect this type of reaction.

**Table 2.** Diaryl ether linkages of phenols with electron-rich halides as reported by Buchwald *et al.*<sup>31</sup>

entry	halide	phenol	product	base	ligand	yield (%)
1				K <sub>3</sub> PO <sub>4</sub>	33	87
2				K <sub>3</sub> PO <sub>4</sub>	33	73

### 1.8 Improvements in Tetrahydroisoquinoline Ring Closure

During the original investigations into the synthesis of BBIQs, only the Bischler-Napieralski cyclization was utilized in the construction of tetrahydroisoquinoline rings. Characterized by refluxing an amide and a Lewis acid (generally POCl<sub>3</sub>) in benzene, the reaction was widely employed, however very little was understood with regard to the mechanism, or control of product formation. Fodor and Nagubundi<sup>32</sup> in 1980 proposed



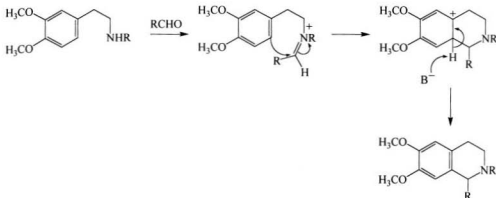
**Scheme 5**

the first accepted mechanism, which has since undergone several revisions and modifications. The mechanism outlined in Scheme 5 is currently accepted.

Generally, the Lewis acid will coordinate to the carbonyl function of the amide to give an iminium salt (**34**). Electrophilic attack by the aromatic ring onto the iminium carbon completes the formation of the tetrahydroisoquinoline ring (**35**). This is followed by the loss of a proton and the elimination of the Lewis base facilitated by the lone pairs on the nitrogen. The resultant iminium ion (**36**) is then reduced by some variation of  $\text{NaBH}_4$ .

A necessary condition for the Bischler-Napieralski reaction is the presence of an electron-donating group *para* to the site of ring closure. Of course, in situations in which there are several electron-donating groups, there are multiple sites for ring closure. Although little is known as to the conditions that control regioselectivity, some investigations give support to both electronic and steric effects. Thus, a desired regioisomer can be favoured through the substitution of either a weakly electron-donating group *para* to an unwanted closing site, or by making one site more sterically hindered than an alternative site.

Since the initial synthetic attempts towards BBIQs, additional methodologies have been employed, primarily the Pictet-Spengler and Pomeranz-Fritsch cyclizations.<sup>33</sup> The Pictet-Spengler cyclisation has quickly risen to become the most popular method for the construction of six-membered nitrogen-containing heterocycles. Characterised by the mechanism shown in Scheme 6, the method is very similar to the Bischler-Napieralski



**Scheme 6**

cyclisation, however, a couple of advantages are immediately apparent: (i) reaction conditions are milder than the traditional Bischler-Napieralski cyclization, being characterized by reacting mixtures of amine and aldehyde in the presence of mildly acidic conditions, either at room temperature or reflux; and (ii) the reaction is “one-pot” leading directly to the formation of the tetrahydroisoquinoline without requiring a subsequent reduction. Generally the aldehyde or aldehyde derivative condenses onto the amine with the remaining steps of the mechanism continuing the same as outlined in Scheme 5.

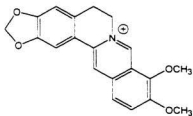
This thesis will describe approaches used by this author towards the total synthesis of oxyacanthine, berbamine, cycleanine and related structures using recently developed methodologies. The use of chiral auxiliaries to control the chiral outcome of the stereogenic centres at C1 and C1' will also be described.

## CHAPTER 2

### BBIQ EXTRACTION

#### 2.1 Introduction

*Mahonia aquifolium* (Berberidaceae) can be found mainly on the Pacific coast of North America and in Central Europe.<sup>34</sup> California Indians originally used the berries as appetite stimulants. Since this initial use other pharmacological activities have been explored, including the use of the berries as aphrodisiacs, diuretics, etc.; the root for alleviating diarrhea, fever, uterosis, etc.; the tincture for treatment of acne, arthritis, bronchitis, etc., and the bark to treat psoriasis and other skin irritations.<sup>1,35</sup> Recent investigations into some of these medicinal remedies have been conducted, however very little evidence or understanding has been gained with respect to the active components or their mechanism of action. A relatively large proportion of *M. aquifolium* is composed of alkaloidal material (3%) with the most abundant constituent being berberine (**36**).<sup>4</sup> Further fractionation of extracts has yielded several BBIQ alkaloids that have been identified as being possible antioxidant and antiproliferative agents, which are suspected to contribute to the medicinal effects of *M. aquifolium*.<sup>4</sup>



**36**



The BBIQs identified in *M. aquifolium* include seven different compounds that are divided into two sub-classes, herein referred to as: (a) the oxyacanthine (**2**) structural class and, (b) the berbamine (**3**) structural class. Of these compounds isotetrandrine (**9**) was the first to be isolated and identified from the root portion of the plant,<sup>36</sup> followed by the isolation of oxyacanthine (0.048%), aromoline (**7**, 0.005%), baluchistine (**37**, 0.074%), berbamine (0.042%), obamegine (**38**, 0.038%), and aquifoline (**39**, 0.071%) in subsequent years.<sup>4</sup> The literature examined to this point indicates the root portion of the plant as having the richest alkaloid content, with few studies on the bark and foliage extractions.

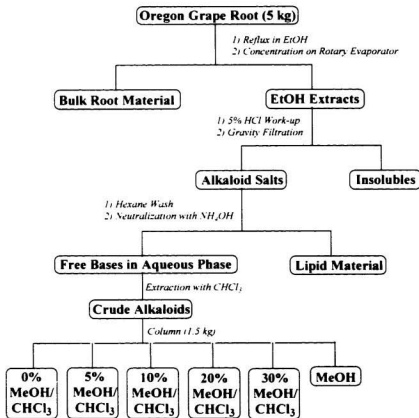
The research described in this chapter focuses on determining specific indicators of biological activity of various alkaloidal extracts. With this knowledge, the intent was to continue the fractionation of these extracts to the point of isolating and identifying specific alkaloids. This would allow activity-compound correlations to be made, identifying one or more alkaloids as exhibiting the pharmacological activity of interest (the methods for determining biological activity will be further expanded upon in future chapters). Based upon published reports, the BBIQs were suspected to be the most active constituents.<sup>7,34</sup> If our findings were in agreement, the intent was to isolate one or more of the compounds, fully characterize the structure(s) and use the data for comparison with future synthetic targets.

## **2.2 Results and Discussion**

### **2.2.1 Progress in the Isolation and Elucidation of BBIQs**

There exists a multitude of methods for the isolation and structural elucidation of

natural products.<sup>37-41</sup> In general, the dry plant material is first extracted with a polar solvent system such as MeOH or EtOH either at room temperature or at reflux. The solvent is evaporated to give a residue, which is dissolved in an acidic medium and washed with a non-polar organic solvent (e.g. hexane), which removes neutral components such as lipids, glycosides, etc. The aqueous layer is then made basic and

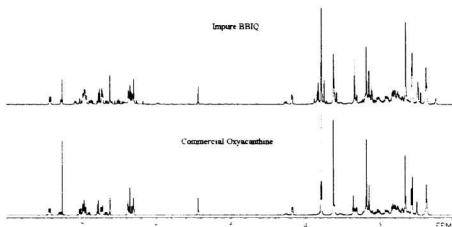


**Figure 2.** Flow diagram of the alkaloid extraction process from Oregon Grape Root.

extracted with another organic solvent, which after drying with a dehydrating agent such as magnesium sulfate, is filtered and again evaporated. A crude alkaloidal extract is thus obtained which can be further fractionated and purified.

The isolation process used in this project followed the general method outlined in Figure 2. The isolation of individual alkaloids was not met with great success. The various extracts were tested for their biological activities using lipoxxygenase inhibition, and keratinocyte anti-proliferation assays, however we were not able to isolate a pure sample of a BBIQ. After several chromatographic separations (FCC & PLC) some fractions compounds were obtained which stained orange with Dragendorff reagent and whose  $^1\text{H}$  NMR spectra revealed peaks characteristic of BBIQ alkaloids. Probably the most promising result is the  $^1\text{H}$  NMR spectrum (Figure 3) of an off-white solid obtained after column chromatography. Figure 3 compares the  $^1\text{H}$  NMR spectra of the crude product with that of commercially-purchased oxyacanthine.

As is evident from the spectra, the signals representing the methoxy protons ( $\delta$  3.79, 3.62, 3.19), *N*-methyl protons ( $\delta$  2.67, 2.57) and the aromatic protons match those of oxyacanthine. This gave a very promising indication of the presence of a BBIQ alkaloid, however the spectra also indicated that impurities were still present. TLC analysis revealed only very small amounts of other substances. Unfortunately, all attempts to crystallize the product have failed. It was then presumed that liquid chromatography mass spectrometry (LC MS) could help to identify the minor components. However, considering the polar nature of these alkaloids and the "tailing" observed in the



**Figure 3.** Comparison of the  $^1\text{H}$  NMR (500 MHz) spectra of an impure BBIQ isolated from *Mahonia aquifolium* and commercial oxyacanthine in  $\text{CDCl}_3$ .

chromatographic procedures used, considerable difficulty was encountered in devising a suitable solvent system that could efficiently separate the components of the mixture. With the consideration that volatile chlorinated organic solvents were to be avoided with the LC/MS, an acetonitrile/water system was the only system which could be employed. One unreproducible run revealed two barely separable peaks having the same molecular mass ( $m/z = 608$ ), suggesting that a mixture of berbamine and oxyacanthine was present, however sufficient evidence to make a direct conclusion could not be obtained.

### 2.3 Summary

Although attempts to isolate several different alkaloids from *M. aquifolium* resulted in the isolation of only one impure BBIQ, experience was gained into the

extraction process as well as of the work, time and costs involved. Nevertheless, the isolation of various alkaloidal fractions for subsequent biological analysis in the lipoxigenase inhibition and DPPH radical reduction assays was accomplished and these assays are discussed in greater detail in the following chapters.

## 2.4 Experimental

### General Section

All organic solvents were redistilled ( $\text{CHCl}_3$  over  $\text{P}_2\text{O}_5$ ,  $\text{CH}_2\text{Cl}_2$ , and hexane over  $\text{CaH}_2$ ), and all other reagents were purchased in the highest chemical grade available from Sigma-Aldrich. Chromatography was performed using 60 mesh silica gel and preparative layer (1 mm) chromatography (PLC) with standard thin layer chromatography (TLC) grade silica gel. Flash chromatography was conducted using 230–400 mesh silica gel.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were obtained on a General Electric GE-300 NB spectrometer at 300 MHz and 75 MHz, respectively, in  $\text{CDCl}_3$  unless otherwise specified.  $^1\text{H}$  shifts are relative to an internal trimethylsilane signal, and shifts in the  $^{13}\text{C}$  spectra are relative to the solvent.

### General Procedure

Oregon Grape root (5 kg) obtained from Global Botannical, Barrie, Ontario was refluxed in ACS grade 95% ethanol over a period of approximately 16 days. The ethanol was repeatedly removed from the root material and evaporated on a rotary evaporator to give a thick black liquid (321 g). This was dissolved in  $\text{HCl}_{\text{aq}}$  (5%), filtered into a separatory funnel and washed with hexane. The resultant aqueous layer was basified with aqueous concentrated  $\text{NH}_4\text{OH}$  and extracted repeatedly with  $\text{CHCl}_3$ . The combined organic layers were dried over  $\text{MgSO}_4$  and evaporated on a rotary evaporator to give a black solid (23 g).

The solid was re-dissolved in  $\text{CH}_2\text{Cl}_2$ , silica (30 g) was added and the solvent was

removed on a rotary evaporator. The resultant product-silica mixture was loaded onto a silica gel column (2.0 kg) and eluted with an increasing polarity MeOH/CH<sub>2</sub>Cl<sub>2</sub> solvent system. The fractions were further purified via flash column chromatography and preparative layer chromatography utilizing some variation of a CH<sub>2</sub>Cl<sub>2</sub>/MeOH/Et<sub>3</sub>N solvent system to yield an off-white solid.

## CHAPTER 3

### LIPOXYGENASE INHIBITION ASSAY

#### 3.1 Introduction

5-Lipoxygenase (LOX) is an enzyme with a catalytic ability to oxidize 1,4-*cis,cis*-pentadiene fatty acids to prostaglandins (PG's) and leukotrienes (LT's).<sup>9,42</sup> These products have been recognized as mediators of inflammation in patients suffering from rheumatoid arthritis, psoriasis, and asthma. Although the mechanism of action is not clearly understood, it is a reasonable assumption that inhibiting the action of lipoxygenase can correlate with anti-inflammatory and anti-proliferative activity in animals and humans.<sup>42</sup>

The lipoxygenase inhibition assay is a relatively simple and inexpensive *in vitro* test for biological activity. Over the past decade, a variety of compounds have been tested as possible LOX-inhibiting agents including anthralin and anthralin derivatives,<sup>41-47</sup> protoberberine alkaloids,<sup>4,5</sup> bisbenzyltetrahydroisoquinoline alkaloids,<sup>9,5</sup> and thiazolyl and benzothiazolyl Schiff bases.<sup>42</sup> Experiments are normally conducted in an incubated chamber where the reaction between a chosen substrate and the LOX enzyme is monitored by the consumption of oxygen in a buffered medium. The dissolved oxygen is measured with a Ag-Pt electrode polarized to between 0.5 and 0.8 volts. The probe is enclosed within a thin plastic membrane and immersed in an aqueous KCl solution. When a voltage is applied to the system, the reaction between the Ag electrode and the chloride ions in the solution generates silver chloride. The electrons generated are used by the platinum electrode to reduce dissolved molecular oxygen that diffuses through the



membrane from the reaction solution. The current that is produced by this reaction is proportional to the dissolved oxygen concentration in the buffered medium.<sup>48</sup>

### 3.2 Results

#### 3.2.1 Comparison of the Inhibitory Effects of Individual Alkaloids and Extracts from *Mahonia aquifolium*

Müller *et al.*<sup>4</sup> and Gallé *et al.*<sup>42</sup> were the first to report on the efficacy of individual alkaloids and extracts from *M. aquifolium* as lipoxygenase inhibitors. They reported the plant extract to be the most effective with an  $IC_{50}$  value of 50  $\mu$ M, followed by oxyacanthine (**2**), berbamine (**3**) and berberine (**36**) in that order, being less effective, having  $IC_{50}$  values  $> 100$   $\mu$ M. Košťálová *et al.*<sup>44</sup> examined the inhibitory effects of some protoberberine, aporphine, and bisbenzyltetrahydroisoquinoline alkaloids in two separate publications. Each paper compared a different set of alkaloids at different concentrations, with a crude *M. aquifolium* extract. The results are presented in Table 3 (Determination

**Table 3.** Lipoxygenase inhibitory properties of alkaloids and extracts from *M. aquifolia* as reported by Košťálová *et al.*<sup>44</sup>

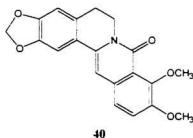
	% Inhibition	
	10 x 10 <sup>-6</sup> (M)	100 x 10 <sup>-6</sup> (M)
oxyberberine ( <b>40</b> )	58	
oxyacanthine ( <b>2</b> )	—	41
berberine ( <b>36</b> )	10	—
berbamine ( <b>3</b> )	—	55
<i>M. aquifolia</i> extract	40	57

of extract concentration is outlined in the experimental).

When the data reported by Müller and Gallé are compared with those of Košťálová, it can be seen that the results with regard to the efficacy of oxyacanthine, berbamine, and the extract are generally in agreement but there is no such agreement when the data obtained with the protoberberine alkaloids are compared. Table 3 indicates berberine as being a poor inhibiting agent of LOX at a concentration of 10  $\mu$ M, although

its efficacy at 100  $\mu$ M was not reported.

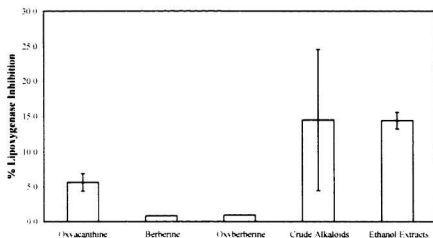
Oxyberberine (**40**) is revealed as exhibiting a very potent inhibitory action at 10  $\mu$ M, an inhibitory effect which is far greater than those of the other test compounds. This is a very surprising result, considering the relative inactivity which was



demonstrated for the non-oxidized berberine structure.

A goal of the research initiatives described in this chapter was to provide an effective comparison of the inhibitory abilities of oxyberberine (**40**), oxyacanthine (**2**), berberine (**36**), a crude total ethanol extract from *M. aquifolium*, and a crude alkaloid fraction derived from the total extract. In addition, interest was particularly concentrated on the action of oxyberberine as having a distinct structural feature which could account for its reported enhanced potency.

The results in Figure 4 represent a repetition (conducted in our laboratory) of the assay conducted by Košťálová's group, in which five test compounds were compared at

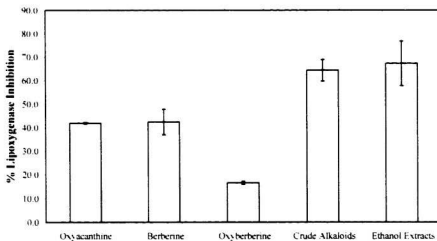


**Figure 4.** Comparison of oxyacanthine (**2**), berberine (**36**), oxysterberine (**40**), crude alkaloids and EtOH extracts from *M. aquifolium* at 10  $\mu$ M. Results are means  $\pm$  S.D. (n = 3).

concentrations of 10  $\mu$ M. Figure 4 reveals the *M. aquifolium* ethanol extract and crude alkaloid fraction to be the most potent LOX inhibitors. These results are of no real surprise and correlated well with the data presented by Košťálová's group. However, there are discrepancies found for the remaining three test compounds. When compared to the results obtained by Müller, Gallé, and Košťálová *et al.*, the berberine result is consistent. However, oxyacanthine exhibited a significant inhibitory effect considering that the concentration used was one tenth of that previously reported. When compared to Košťálová's results alone, a significantly lowered effect is witnessed with each compound. This is especially noticeable in the protoberberine alkaloids (**36**, **40**) where our results show that there is virtually no LOX inhibition, particularly in the case of oxysterberine.

Nonetheless, consideration must be given to the large standard deviation represented in Figure 4. The data were very difficult to reproduce, especially with regard to the alkaloid extracts. Also, standard deviation could not be calculated accurately for the protoberberine alkaloids considering that several of the trial runs did not show any inhibition. In an effort to circumvent this problem, the experiment was repeated at higher concentrations.

Figure 5 reveals the % LOX inhibition for the five test compounds at 20 mM, solutions which are 2,000 times more concentrated than those reported in the previous experiment. Several observations are immediately noticeable from the data: 1) there are smaller standard deviations; 2) the potency of berberine and oxyacanthine is the same; 3)



**Figure 5.** Comparison of oxyacanthine (**2**), berberine (**35**), oxyberberine (**39**) and the EtOH extracts and crude alkaloids from *M. aquifolium* at 20 mM. Results are means - S.D. (n=3).

oxyberberine was the least potent alkaloid tested. These results question the validity of oxyberberine as a potent inhibitor of anti-inflammatory activity previously reported by Košťálová *et al.*. However, the fact that berberine was more potent at a concentration 2,000 times greater than that which was observed in the previous experiment is also significant and warrants further consideration.

### **3.2.2 Dose-dependency of Individual Alkaloids and Extracts from *Mahonia aquifolium***

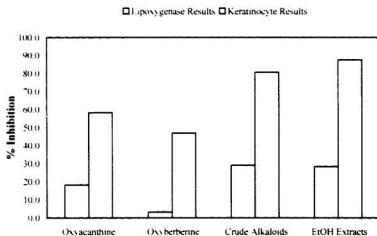
Considering the vast difference in the concentrations of the two previously reported experiments, it was decided that a study of the dose-dependency of the test compounds was warranted. Figures A1-5 illustrate the dose-dependency for oxyacanthine, berberine, oxyberberine, the crude EtOH extracts and alkaloid fractions, respectively.

The data indicate a clear linear dose-response for each of the test compounds. The inhibitory ability correlates best with Figure 4, namely in the order: ethanol extracts > crude alkaloids > oxyacanthine > berberine > oxyberberine. It should be noted, that the ethanol extracts and crude alkaloids demonstrated the largest standard deviation, a trend that has been evident in each of the experiments.

### **3.2.3 Keratinocyte Inhibition Assay**

As previously described, leukotrienes are potent mediators of inflammation originating from the oxidation of fatty acids. These compounds serve to stimulate keratinocyte proliferation, resulting in scaling and plaque formation of the epidermis.

unsightly symptoms characteristic of psoriasis. Considering the closely related mechanisms of inflammation and proliferation, complimentary assays have been developed that examine the ability of various test compounds to inhibit keratinocyte cell growth. Müller *et al.*<sup>46,47</sup> examined the abilities of various anti-inflammatory agents to inhibit the proliferation of HaCaT cells, which have been reported as being models of epidermal hyper-proliferation in psoriasis. In addition, Müller *et al.* also examined the abilities of oxyacanthine, berbamine, berberine and extracts from *M. aquifolium* to act as anti-proliferative agents. They found that the results that they obtained in this particular assay were the reverse of what they found for the anti-inflammatory activities. The BBIQs oxyacanthine and berbamine were found to be the most potent, while berberine



**Figure 6.** Comparison of the abilities of oxyacanthine (**2**), oxyberberine (**40**), crude alkaloids and EtOH extracts from *M. aquifolium* to inhibit cell growth and the action of lipoxigenase (100  $\mu$ M).

and *M. aquifolium* extracts were found to exhibit only moderate activity.

This thesis also describes an account of the abilities of oxyacanthine, oxyberberine, crude alkaloids and EtOH extracts to inhibit HaCaT cell growth. This portion of the research was performed in collaboration with and in the laboratory of Dr. S. Kaiser of the Faculty of Medicine, Memorial University of Newfoundland. Figure 6 compares the magnitudes of LOX inhibition with anti-proliferative activity, although insufficient data had been obtained at the time of writing this thesis to determine standard deviations. Furthermore, it should be noted that this work represents a fairly recent and still ongoing investigation. Repeat assays of the test compounds including berberine are

**Table 4.** Müller's results indicating anti-proliferative activity as an  $IC_{50}$  value.

	$IC_{50}$ ( $1.0 \times 10^{-6}$ M)
<i>M. aquifolium</i>	35
berberine	30
berbamine	11
oxyacanthine	13

still in progress.

According to the data represented in Figure 6, there is correlation in the order of inhibitory activity (EtOH extracts > crude alkaloids > oxyacanthine > oxyberberine), but the absolute magnitudes of inhibitory activity is more pronounced in the anti-proliferation assay. Although the results indicate a positive correlation between the two protocols, they do not agree with the observations of Müller *et al.*, whose results are presented in Table 4.

They reported efficacy as the concentration at which 50% of HaCaT cell growth was inhibited ( $IC_{50}$ ). When the data which is presented in Figure 6 is treated in a similar manner, the constituents of *M. aquifolium* appear to have relatively low anti-proliferative activity.

However it should be noted that when the cells were counted after incubation, some of the cells in the wells inoculated with the crude alkaloids and EtOH extracts appeared not to be viable. Thus, these compounds might have actually killed the cells, rather than have inhibited their proliferation. No definitive conclusions could be made without cell viability tests which were not completed at the time of writing this thesis.

### **3.3 Discussion**

#### **3.3.1 Comparison of Studies**

At the onset of this investigation, an attempt was made to bridge some of the discrepancies in the results reported by the groups of Müller, Galle and Košťálová. This was accomplished for the determination of the order of efficacy of the *M. aquifolium* extracts and constituents, but not for determining the actual magnitudes of efficacy. The results suggest that the ability of inhibitory activity is represented in the following order: EtOH extracts > crude alkaloids > oxyacanthine / berberine > oxyberberine. In comparison to the values reported by the aforementioned authors, those reported in this thesis are generally smaller. A more significant fact was the observation of the virtual lack of a LOX inhibitory effect by oxyberberine (**40**), a result that directly contradicts



those obtained by Košťálová *et al.*

Previous suggestions for structure-activity correlation have concentrated on the lone pairs of electrons on the nitrogen in protoberberine alkaloids,<sup>4</sup> and on the phenolic groups in the oxyacanthine-berbamine structures.<sup>4,5</sup> It is believed that these functional groups serve to inactivate hydroperoxides, thus removing one of the components necessary for the oxidation of fatty acids, effectively shutting down the activity of lipooxygenase in the *in vitro* assay. If this is the case, then the mechanism of action may be the same as the mechanism for quenching stable free radicals such as DPPH (to be further expanded upon in the following chapter).

### **3.3.2 Investigations into the Activity of the Extracts From *M. aquifolium***

Overall, the EtOH extracts and crude alkaloid fractions proved to be the most effective, which requires consideration of the following questions:

- 1) What is responsible for the inhibitory action of these mixtures?
- 2) If the alkaloids are responsible, is there an agonistic effect?
- 3) If the BBIQ or protoberberine alkaloids are not active, what is?

With respect to the first question, Košťálová *et al.* attempted to provide a thorough analysis of the attributing effects of the individual BBIQ alkaloids (oxyacanthine (**2**), berbamine (**3**), aromoline (**7**), baluchistine (**37**), obamegine (**38**), aquifoline (**39**)), as well as the protoberberine and aporphine alkaloids isolated from *M. aquifolium*. As previously discussed, they found that berbamine and oxyberberine exhibited a potency that was either equal to or greater than that of the crude extracts which they examined. Considering that

their results were not consistent with those reported herein, or those of Müller, or Galle. another aim of this study considered examining alternative hypotheses.

This led to investigations of a possible agonistic effect amongst the alkaloids. In their studies on the anti-inflammatory action of *Berberis vulgaris* root extracts, Ivanovska and Philipov<sup>42</sup> attempted to provide insight into this topic by performing various *in vivo* and *in vitro* tests on root-derived alkaloid fractions, as well as on some isolated individual alkaloids. Their results were inconclusive as to which extract constituents exhibited the greatest effects. We therefore attempted to repeat this type of investigation with *M. aquifolium*, testing various alkaloid fractions obtained from column chromatographic separation for their abilities to inhibit the action of lipoxigenase. By successive fractionation of the alkaloids in the tested fractions, one could start to narrow in on which compounds were individually active, and which compounds were active as mixtures. The results however, obtained from this set of experiments were also inconclusive. There was no significant difference in activity from the original ethanol extracts and crude alkaloids to various chromatographically separated fractions.

To answer the third question, results obtained from the keratinocyte anti-proliferation assays must also be considered. As discussed in Section 3.2.3, inoculation of the cells with the ethanol extract and crude alkaloids resulted in what appeared to be cell death. If this were the case, then the results do not represent a true inhibitory effect. This consideration could also be applied to observations derived from the LOX inhibition assay. The total extracts and crude alkaloids are mixtures of many compounds, some of

which have direct inhibitory activity, while others do not. It is conceivable that such compounds may serve to interfere in the efficacy of active constituents, or to perturb the oxidation reaction by means not yet considered. Therefore, the values reported for these extracts may not accurately represent the cumulative sum of the action of its individual constituents.

### **3.4 Summary**

In summary, the crude alkaloids and total ethanol extracts from *M. aquifolium* have been shown to be the most potent lipoxygenase inhibiting agents. These are followed by the activity of the individual alkaloids oxyacanthine (**2**), berberine (**36**), and oxyberberine (**40**). Preliminary studies into structure-activity correlations lend support for the presence of a phenolic group on the oxyacanthine structure being an important requirement for activity, with no definitive conclusions as to the action of the protoberberine alkaloids, oxyberberine and berberine. Furthermore, no such correlation has currently been provided to explain the action of the crude alkaloids and extracts. In addition, further studies into anti-inflammatory activity are currently being investigated through the antiproliferation assays.

### **3.5 Experimental**

#### **General Section**

All microgram mass determinations were measured on a CAHN 27 Automatic Electrobalance. Oxygen consumption was measured with a Clark electrode in a water-incubated chamber. All reagents and solvents used were of the highest grade available from Sigma-Aldrich. Oxyacanthine sulfate was purchased from Carl Roth Pharmaceuticals (Karlsruhe, Germany) and all other solutions and/or compounds were prepared as described herein.

#### **Linoleic Acid Substrate Preparation**

To borate buffer (2.0 mL, 0.1 mol/L, pH = 9.0) in a 50 mL Erlenmeyer flask was added Tween 20 (100  $\mu$ L). To this mixture was added linoleic acid (100  $\mu$ L) with continuous stirring, the resultant emulsion was cleared by the addition of NaOH (0.26 mL of 1.0 mol/L). The solution was then diluted and adjusted to pH 6.5 with buffer (9.0 mL) and distilled water (10 mL).

#### **Lipoxygenase Enzyme Solution Preparation**

Lipoxidase containing 15,000,000 units of protein (46,000 units  $\cdot$  mg of solid) was purchased from Sigma. Solutions were prepared by dissolving 1.0 mg of lipoxidase in 5.0 mL of borate buffer.

#### **Test Compound Solution Preparation**

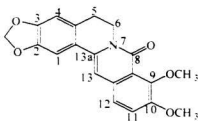
Inhibitors were dissolved in 10.0 mL of DMSO at the required concentrations. Slightly insoluble compounds were mixed by sonication. Concentrations of *M*.

*aquifolium* extracts were calculated on the basis of the quantity of berberine, as determined by HPLC analysis.

### General Assay Procedure

All experimental runs were performed in triplicate with oxygen consumption being expressed on a strip chart recorder. The strip chart recordings from the inhibition experiments were measured and compared to that provided by an initial blank comprised of substrate, enzyme and DMSO. To the electrode chamber was added 1.5 mL of substrate followed by the simultaneous addition of lipoxxygenase (200  $\mu$ L) and inhibitor (200  $\mu$ L). The experimental runs were conducted over a period of 240 s at a constant temperature of 298 K.

### 5,6-Dihydro-9,10-dimethoxy-2,3-methylenedioxy-8H-dibenzo[a,g]-quinolizin-8-one (oxyberberine)<sup>72</sup>



A solution of berberine chloride (0.50 g, 1.34 mmol) in water (15 mL) was brought to a gentle reflux followed by cooling to, and maintaining at 80 °C. At this point, a solution of KOH (9.0 g) in water (5 mL) was added. A gummy solid was immediately formed. After cooling to room temperature, the crude product was collected by filtration, pulverized and then returned to the original solution. The mixture was stirred vigorously and refluxed for an additional 10 h. The solution was cooled to room temperature, filtered and the crude product was purified by silica gel column chromatography using 5 % MeOH/CHCl<sub>3</sub> as the eluant to give oxyberberine as a

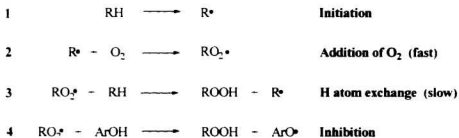
yellow powder (0.172 g, 37 %): mp 204 °C (lit.<sup>73</sup> mp 204 °C); IR (neat  $\text{CDCl}_3$ ):  $\nu_{\text{max}}$  1655 ( $\text{cm}^{-1}$ );  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.30 (d,  $J = 5.2$  Hz, 2H, H-11, H-12), 7.21 (s, 1H, H-13), 6.72 (s, 1H, H-1), 6.71 (s, 1H, H-4), 6.02 (s, 2H,  $\text{OCH}_2\text{O}$ ), 4.30 (t,  $J = 6.0$  Hz, 2H, H-6), 4.02 (s, 3H,  $\text{OCH}_3$ ), 3.96 (s, 3H,  $\text{OCH}_3$ ), 2.90 (t,  $J = 6.0$  Hz, 2H, H-5); MS ( $m/z$ ) (% intensity): 351 ( $M^+$ , 100), 336 (73), 322 (28), 308 (22).

## CHAPTER 4

### DPPH RADICAL-SCAVENGING ASSAY

#### 4.1 Introduction

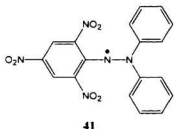
Although it is a necessity of life, oxygen also has the potential to cause great harm in biological systems. Radical species, generated by the action of ultraviolet or other ionizing radiation, chemicals, enzymes or by electron leakage in the course of metabolic pathways,<sup>6</sup> have been implicated in a multitude of various disorders including: asthma, arthritis, inflammation, neurodegeneration, Parkinsons disease, and dementia.<sup>6</sup> These oxygen atom-containing radicals directly attack bio-molecules (proteins, nucleic acids, polyunsaturated lipids, sugars) altering their properties, and disrupting the function and structure of cells.<sup>6,43</sup> A likely reaction series demonstrating radical generation is shown in Figure 7.<sup>14</sup>



**Figure 7.** Steps 1-3 represent the oxidation series believed to occur in biological systems. Step 4 represents inhibition by various hydrogen donating compounds.

Upon generation of the free radical (Step 1) (RH = protein, nucleic acid, lipid molecule, etc.) a rapid addition to molecular oxygen results in the formation of a hydroperoxide radical (Step 2) which undergoes a slower, rate-determining step to attack another bio-molecule (Step 3).<sup>54</sup> In order to prevent the continuation of this cycle, compounds must be utilized that can either prevent the radical from being generated, or "scavenge" the radical before it has had the opportunity to react again in the third step ("chain-breaking antioxidant"). Most antioxidant compounds reported in the literature contain one or more phenolic functions in their structures, which allow them to quench the offensive radical through hydrogen donation (Step 4).<sup>54-57</sup> In order for these scavenging radicals to be effective, they must be relatively stable so as not to attack the substrate, but at the same time they must be reactive enough to interact with the radical compound that they are scavenging.<sup>55</sup> If the reaction in Step 4 is sufficiently exothermic, then the energy barrier to the transfer of the hydrogen atom decreases sufficiently to allow for an effective reductive process.

A variety of physical, chemical and biochemical methods have been utilized to test the ability of various compounds to inhibit this oxidative process.<sup>58</sup> One common



method involves the reduction of the stable free

radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, **41**).<sup>6</sup>

This compound forms a deep-violet solution, which loses its colour upon reduction to the corresponding hydrazine. This subsequent decrease in absorbance



can be monitored in the visible region ( $\lambda = 517 \text{ nm}$ ) using a uv-vis spectrophotometer, allowing for the determination of the rate constants and stoichiometry of the scavenging reaction.

An objective at the outset was to test the ability of various components of *M. aquifolium* to interact with the DPPH. However, considering the scarcity of data on the mechanism of antioxidant effects of BBIQs in general, it was decided to evaluate the hydrogen-donating abilities of alternative functionalities that are present in these compounds. Since oxyacanthine and berberine were the only constituents of *M. aquifolium* that could be procured from commercial sources, it was decided to employ these compounds in detailed rate studies with DPPH.

## 4.2 Results

### 4.2.1 Müller's Reports

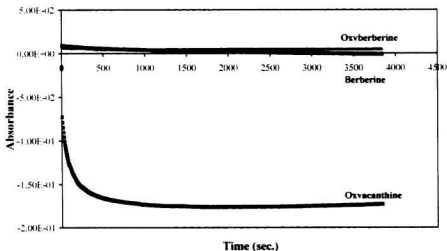
The work conducted by Müller *et al.* on extracts from *M. aquifolium* has generated a great deal of interest. While concentrating on the analysis of anthralin and its

**Table 5.** Rate constants for the reduction of DPPH as reported by Müller *et al.*

	$k_{\text{DPPH}} (\text{M}^{-1} \text{s}^{-1})$
<i>M. aquifolia</i>	$34.1 \pm 5.3$
berberine	$2.8 \pm 0.6$
berbamine	$115 \pm 1.3$
oxyacanthine	$111 \pm 5.3$
anthralin	$24.2 \pm 4.2$

derivatives. Müller also examined the antioxidant abilities of oxyacanthine (**2**), berberine (**36**), berbamine (**3**), as well as alkaloidal extracts from *M. aquifolium*. Their results were expressed as rate constants and are reproduced in Table 5.

The reaction was reportedly performed in a 1:1 PBS/acetone solvent at 25 °C using equimolar concentrations of test antioxidant and DPPH (0.1 mM). The rate constants were reportedly determined from the slopes of straight line plots of  $1/[DPPH]$  vs. time (s), but exact details are not provided.<sup>44</sup> In order to use this protocol, Müller's experimental conditions were employed with anthralin, *M. aquifolium* crude alkaloids, berberine, oxyberberine, and oxyacanthine. Figure 8 reveals the plots of DPPH absorbance vs. time.



**Figure 8.** Plots of the decrease in absorbance of DPPH in response to addition of oxyacanthine, oxyberberine, and berberine in PBS/acetone (1:1). Concentrations of DPPH and antioxidant were equimolar (0.1 mM). Results are expressed as means (S.D. = - 0.04, n = 3).

for the latter three compounds.

The effect of the crude alkaloids and anthralin on the ability to reduce DPPH, resulted in only uninterpretable data. For anthralin similar results were attributed by Müller to the fact that a very unstable anthralin radical was formed, which readily dimerizes to bianthrone, a compound with markedly less radical scavenging ability than its monomeric form. This side reaction is a common one and was examined in detail by Müller and his group.<sup>45</sup> The crude alkaloids on the other hand, themselves produced deeply coloured solutions, thereby interfering with the spectrophotometer's ability to measure any meaningful decrease in absorbance associated with the quenching of DPPH. Also, the crude alkaloid extract is a mixture of many compounds, some of which may serve to inhibit the interaction between various active components and the DPPH radical.

Oxyberberine (**40**) and berberine (**36**) are poor reducing agents of DPPH. These compounds lack phenolic or other functionalities that are known to be effective for hydrogen donation.<sup>46</sup> These observations agree with those reported by Müller *et al.*, but our results with oxyacanthine did not. Müller *et al.* reported that the DPPH radical scavenging reaction of each of these compounds obeyed second order kinetics. This is a reasonable assumption, considering that most functional groups capable of quenching the DPPH radical follow this trend.<sup>46, 90</sup> However, our results and the fact that no attempts to determine the stoichiometry were reported, prompted a re-evaluation of Müller's rate constants. As previously mentioned, the plots of  $1/[DPPH]$  vs. time were reported by Müller *et al.* to give straight lines for each of the compounds, from which they obtained

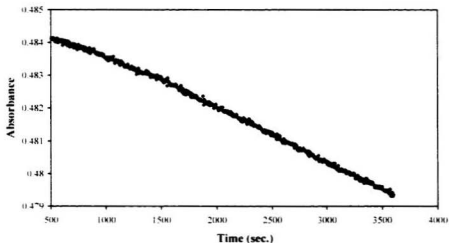
the slopes and rate constants. According to the data presented in Figure 8, berberine (**36**) and oxyberberine (**40**) give straight lines of zero slope, but oxyacanthine (**2**) gives a very pronounced curve. Furthermore, Müller *et al.* proposed that the phenolic group is the only functional group which can contribute an appreciable effect to account for their results.

One of the most disturbing problems involved reproducibility of the data collected using the procedure described by Müller. After considerable experimentation in our laboratory, we incurred the following problems:

- 1) The DPPH is not stable in the PBS/acetone solution. After approximately 1 h a change in the colour of the solution (purple - brownish) was observed which could be monitored by uv-vis spectrometry (Figure 9).
- 2) The 1:1 PBS acetone solution is not a suitable buffering system. The phosphate crystals precipitated out of solution resulting in a decrease in pH from 7.4 to approximately 6 over a period of 1 h.
- 3) Müller reported using equimolar DPPH and test compound, which is not appropriate for calculating accurate rate constants. As will be discussed later, a more appropriate method requires that the antioxidant be the limiting reagent.
- 4) The time-frames or intervals required for data acquisition are critical. Müller did not elaborate on this aspect.

As a result of these problems, changes to the assay procedure were made in order to obtain more reproducible data. The first consideration related to the spontaneous rate of decay of the DPPH radical itself, since if it changes colour during the one hour period

without any antioxidant present, it would be difficult to get an accurate indication of the activity of our test compounds alone. This rate of decay of the DPPH could be potentially significant for determining accurate rate constants of poor radical scavenging compounds.



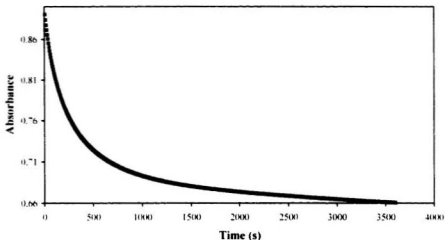
**Figure 9.** The natural decay of DPPH over 3600 s in a PBS acetone buffered medium.

since Figure 9 reveals that there is only a small rate of decay as measured by the DPPH absorbance change. In order to compensate for changes in the absorbance, a blank containing all but the antioxidant was used with a dual beam uv-vis spectrophotometer. Therefore, the natural DPPH absorbance decay in the blank could be subtracted from the experimental absorbance decay in the sample. Although the reproducibility of the results was improved, a couple of problems still existed. Because both the reference and the

sample solutions were coloured, the initial absorbance reading was zero as expected, but since the radical was being destroyed, the absorbance of the test solution decreased relative to the blank. Therefore data was recorded as a negative absorbance difference. The use of negative absorbance values in subsequent calculations proved to be inconvenient, as was the requirement to have to prepare fresh reagent media due to the relative instability of the PBS acetone solution.

A literature search was undertaken to find a better buffered system than PBS acetone. The 2:3 Tris EtOH system reported by Terada *et al.*<sup>17</sup> which allowed the solution to be buffered at a pH of 7.4, and also provided a homogeneous DPPH solution that was stable for up to 24 h was chosen. This solvent system permitted data to be acquired over periods of 1 to 2 h and only required the preparation of one DPPH solution per day, an advantage that was not afforded with the PBS acetone buffered system. All reactions were incubated at 298 K with a water-thermostated cuvette holder during the spectrophotometric determination in which measurements were taken at 6 s intervals and were recorded by computer.

With the more stable Tris EtOH buffered solvent system, the decay of DPPH, or any fluctuations in the pH of the test solutions were negligible. This enabled a reference composed of oxyacanthine and solvent, without the presence of DPPH, to be used. Considering that the sample solution containing DPPH is initially coloured, a decrease in absorbance with reference to the non-coloured blank resulted in absorbance readings having positive values (Figure 10). Figure 10 reveals the rapid decrease in absorbance



**Figure 10.** Plot of the decrease in absorbance of DPPH in response to addition by oxyacanthine. The decrease in absorbance was measured against a blank of solvent and oxyacanthine. The DPPH : oxyacanthine molar ratio was 5:1 respectively for this assay. Results are expressed as means (S.D. = - - 0.003, n = 3).

observed in the Tris EtOH system. However, unlike the results obtained previously, data obtained with this system were reproducible (S.D. = - - 0.003). An important fact to be noted is that 5:1 molar ratios of DPPH : oxyacanthine were employed. With oxyacanthine being the limiting reagent, the rate constants and stoichiometry could be calculated, which was not possible using Müller's method. A more detailed discussion of the reaction kinetics as well as buffer solvent effects will be expanded upon in further sections.

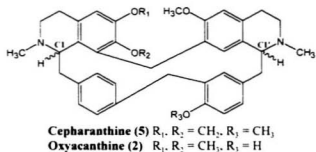
#### **4.2.2 Alternative Functional Groups for DPPH Reduction**

The sharp decrease in absorbance revealed in Figure 10 represents a relatively rapid reductive process occurring during the first 10 min. These results raised questions

as to the kinetics of the reaction and whether the entire effect can be attributed solely to the action of the single phenolic functional group in the molecule.

A literature search revealed a variety of test compounds that could reduce DPPH and act as antioxidants, however most of them contained at least one phenolic group. McGowan *et al.*<sup>64</sup> reported studies of several amines as effective radical scavengers. They concluded that primary and secondary arylamines were effective scavengers, but that tertiary amines lacking hydrogen atoms on the nitrogen were not. In 1999 Terada *et al.*<sup>17</sup> reported the effects of pH on the reductive abilities of cepharanthine (**5**).

As can be seen from **5** and **2**, cepharanthine and oxyacanthine are structurally similar and differ only in the substitution at R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> and have the opposite stereochemical configurations at C1 and C1'. The lack of a phenolic group on cepharanthine suggests that it should be a very poor antioxidant relative to oxyacanthine. In fact, this was demonstrated to be the case in earlier studies by Nagatsuka and



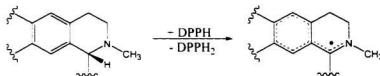
Nakazawa.<sup>70</sup> Any effect that cepharanthine exhibited towards the inhibition of antioxidant activity was believed to be limited to membrane stabilization. Considering



the relatively large and bulky structure of the BBIQ skeleton. Nagatsuka and Nakazawa assumed that cepharanthine inserted into the phospholipid bilayer, exhibiting a similar function as that of cholesterol. However, when measuring the ability of cepharanthine to reduce the DPPH radical, they used an acidic medium (pH = 5.5) instead of a medium having the physiological pH of 7.4 as is more typically found in biological systems.

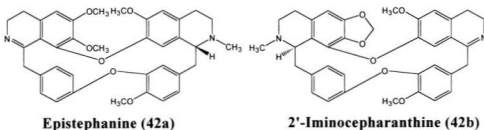
Terada *et al.* recognized this limitation and proceeded to examine the effects of pH on the DPPH-cepharanthine reaction, and reported results which were different from previous considerations of BBIQs. They found, that in buffered acidic medium (2:3 MES:EtOH, pH = 5.5), cepharanthine exhibited no reductive or radical trapping effects on DPPH. However, when the pH was raised to 7.4 (2:3 TRIS:EtOH) there was a dramatic increase in the ability of cepharanthine to quench the DPPH free radical. Terada *et al.* proposed that other groups besides hydroxyl groups could donate a hydrogen radical and thus, allowed the consideration of an alternative mechanism for the action of BBIQs with DPPH.

Terada *et al.* proposed the mechanism depicted in Scheme 7 in which the hydrogen atom on the stereogenic carbon (C1') is abstracted by the DPPH radical. They offered support for this mechanism by suggesting that the lone pair on the nitrogen may



**Scheme 7**

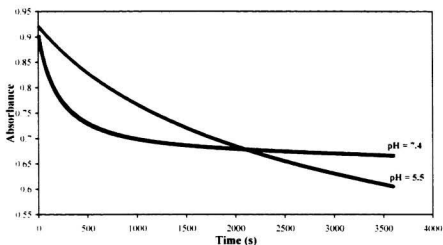
donate electrons to the C1' site, making hydrogen radical abstraction more favorable. At acidic pHs (pH = 5.5) the tertiary amines are protonated to form the corresponding quarternary ammonium salts, as a result of which the nitrogen becomes less able to donate electrons to the C1' position, thereby inhibiting hydrogen abstraction. It should be noted however, that Terada *et al.* considered only the C1' position as being a probable H-atom donor and not the C1 position, support for this consideration coming from lipid peroxidation studies of the 2-imine analog epistephanine (**42a**) and 2'-imine cepharanthine (**42b**). While epistephanine did exhibit lipid peroxidation inhibitory activity, its 2' counterpart (**42b**) did not, thus lending evidence to the fact that only the C1' position



should be considered for H-abstraction. No further rationale for these observations was provided. It must be considered that **42a** has methoxy groups substituting the C1 tetrahydroisoquinoline aromatic ring, whereas cepharanthine has a bridging methylene. Although this may seem to be an insignificant difference, computer molecular modeling using Spartan Pro indicates that this difference may indeed contribute to altering the environment of the C1 and C1' hydrogens.

On the other hand, oxyacanthine has an additional site for radical-scavenging.

With the presence of the phenol, there are two possible sites from which hydrogen abstraction can occur in accordance with Terada *et al.*'s hypothesis. In principle, acidic pH's may affect the ease of H-abstraction from Cl', but there should be no comparable effect on the ease of H-abstraction from the phenol. Therefore, using Terada's pH conditions with oxyacanthine should result in some radical scavenging activity in the



**Figure 11.** Plot of decrease in absorbance as a result of DPPH radical scavenging by oxyacanthine at pH = 5.5 (2:3 MES:EtOH) and pH = 7.4 (2:3 Tris:EtOH). Results are expressed as means (S.D. =  $\pm$  0.003, n = 3).

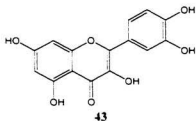
acidic region, and a pronounced effect at pH 7.4. This hypothesis proved to be correct and as shown by the results depicted in Figure 11, there is an accelerated rate of decrease in absorbance at pH = 7.4 and a much slower rate (presumably due to the action of the phenol) at pH = 5.5.

Although the visual representation of the data indicates that the assumptions made

by Terada *et al.* are reasonable, only after an examination of the kinetics could a definitive conclusion be made. Such an endeavour has not been reported by others for BBIQs and efforts were undertaken to define a suitable mathematical representation and hopefully provide definitive evidence for the existence of Terada's, or a more appropriate alternative mechanism.

#### 4.2.3 Reaction Kinetics and Stoichiometry

As shown in Figures 8, 9 and 10 (pH = 7.4), there is a rapid decrease in absorbance in the first 200 s of the reactions depicted, followed by a slowdown and levelling out effect over the last 3,400 s. For compounds with multiple radical scavenging functionalities such as flavonoids (*e.g.* quercetin (**43**), which contain up to 5 phenolic groups), the initial period of radical-scavenging normally corresponds to the abstraction of



the most labile hydrogens, followed by a period that coincides with the formation of degradation-oxidation products.<sup>56</sup> Once a radical has been generated from an antioxidant, a variety of side reactions can occur. Examples include the formation of quinones from phenol-generated

radicals,<sup>47</sup> reactions with other peroxide radicals,<sup>61</sup> or dimerization, depending on the antioxidant.<sup>47</sup>

Dangles *et al.*<sup>43</sup> reported a very detailed analysis of the stoichiometry and kinetics of the reaction between DPPH with quercetin (**43**).<sup>56,62,63</sup> By their analysis using equation

(1), the number of hydrogens (“ $n$ ”) abstracted from the antioxidant (“AH”) by DPPH radicals could be determined from a plot of absorbance vs. time. Using  $A_f$  as the final absorbance,  $A_o$  as the initial absorbance,  $C_o$  as the initial DPPH concentration and  $C$  as the initial antioxidant concentration, the value of  $n$  could be calculated. However, in order for the model to apply, the value of  $C_o/C$  must be larger than  $n$  (for a detailed analysis into the derivation of equation (1), refer to Appendix 2).<sup>64</sup>

Considering the above equation, Dangles *et al.*<sup>65</sup> determined that an antioxidant

$$n = \frac{C_o}{C} \left[ 1 - \frac{A_f}{A_o} \right] \quad (1)$$

with stoichiometry  $n$  is simply modeled as having  $n$  independent functional groups, each with the ability to donate a H-atom with the same second order rate constant  $k$ . With this consideration, it was assumed that plots of  $1/[DPPH]$  vs. time over the initial 60 s would give straight lines from which one could calculate an overall rate constant.

This type of analysis was undertaken in our laboratory with oxyacanthine.

Dangles *et al.*<sup>63</sup> reported calculating the value of  $k$  over the initial 60 s of their reaction, and calculating  $n$  over a 10 min interval for their compounds. If Terada’s hypothesis for the tetrahydro- isoquinoline radical quenching site holds true, then calculation of stoichiometry should give values that are approximately “2”: i.e., one hydrogen corresponding to the C1’ hydrogen of the tetrahydroisoquinoline and one for the hydrogen of the phenol group. Also, because the stoichiometry of an antioxidant corresponds to  $n$

independent functional groups with the ability to donate a H-atom with the same rate constant  $k$ , the total rate constant for an antioxidant should be  $nk$ . Table 6 shows the results obtained in the present study for the action of oxyacanthine at pH = 7.4 and 5.5.

**Table 6.** Values of  $k$  determined from the slopes of  $1/[DPPH]$  vs. time (s) over the initial 60 s. Values of  $n$  were calculated using equation (1). Values for  $n$  and  $k$  were obtained using the observed initial absorbance ( $A_{0,obs}$ ) and the calculated initial absorbance ( $A_{0,cal}$ ).  $R^2$  values obtained for the lines of best fit for the initial 60 s of the reaction.

	$A_{0,obs}$		$A_{0,cal}$	
	pH = 5.5	pH = 7.4	pH = 5.5	pH = 7.4
$k$ (60 s)	1.29 ( $\pm$ 0.16)	10.69 ( $\pm$ 0.10)	37.45 ( $\pm$ 1.68)	61.89 ( $\pm$ 0.83)
$n$ (600 s)	0.54 ( $\pm$ 0.04)	0.95 ( $\pm$ 0.01)	1.57 ( $\pm$ 0.03)	1.87 ( $\pm$ 0.01)
$n$ (3600 s)	1.66 ( $\pm$ 0.05)	1.24 ( $\pm$ 0.01)	2.43 ( $\pm$ 0.01)	2.08 ( $\pm$ 0.02)
$R^2$	0.99	0.99	0.32	0.46

According to the data presented, the conclusion can be made that the rate at which oxyacanthine scavenges free radicals is markedly less than that previously reported by Müller *et al.* (refer to Table 5). This posed a source of concern considering that the results for oxyacanthine compared to other known antioxidants such as flavanoids<sup>(n2,n3)</sup> and anthralin<sup>11</sup> are much lower. In addition, the values of  $n$  which were hypothesized to be “2” according to Terada’s mechanism, turned out not to be the case. Using  $A_0$  as the observed initial absorbance at  $t = 6$  s (0.90), the value of  $n$  was found to be more in the range of approximately “1” for pH = 7.4. However, at pH = 5.5 a very confusing discrepancy was found. A value of “0.54” was determined for the initial 10 min and a value of “1.66” for the entire 60 min cycle. This indicated that, although the rate was

markedly slower, more radical was apparently being scavenged over the indicated time period than at the physiological pH of 7.4. This trend held true for the calculations of  $n$  using a "calculated"  $A_o$  (1.18), but the values obtained agreed more closely to the hypothesized results.

### 4.3 Discussion

In their attempt to find novel compounds exhibiting antioxidant activity, Müller *et al.* reported results for oxyacanthine that we now believe to be unreliable on the basis of several discrepancies (Section 4.2.1). Although our results do not agree with the reported rate constants, we are in agreement that the presence of a phenolic group on the BBIQ structure does contribute to the overall radical scavenging ability of oxyacanthine. Terada's proposed mechanism for C1' H-atom abstraction suggested an alternative functional group on the BBIQ that may enable free radicals to be scavenged. However, at the point of conclusion of this project, despite the fact that no further evidence has been provided to support the mechanism proposed, our results are consistent with Terada's proposal that there is an additional site for radical scavenging on the BBIQ structure.

#### 4.3.1 Significance of " $n$ " and " $k$ " values

According to Terada's rationale, the formation of a quaternary ammonium salt would not permit resonance stabilization of the radical at C1' by the lone pair of electrons on the N2' nitrogen atom. However, there is no logical reason to exclude radical stabilization on what is also a benzylic position, with resonance stabilization involving the aromatic ring of the tetrahydroisoquinoline skeleton. Therefore, H-atom abstraction from

this position could still be favourable. By repeating Terada's protocol with oxyacanthine instead of cepharanthine and using Dangles methodology we were able to obtain a model allowing for a determination of the reaction stoichiometry and rate constants. Since the exact reaction conditions of Terada were used with oxyacanthine, it was anticipated that  $n = 2$  at a pH of 7.4 (corresponding to the hydrogen at the C1' position, and the phenol), and that  $n = 1$  (corresponding to the single phenol group) at a pH of 5.5.

Upon first examination of Table 6, the values of  $n$ , calculated using the initial absorbance ( $A_{0,obs}$ ) during the first 600 s at both pH's, were smaller than predicted. However, the value of  $n$  at pH = 5.5 after 3600 s was closer to the value of  $n = 2$  predicted for pH=7.4. This would serve to indicate that radical scavenging is not confined solely to the action of the phenolic group on oxyacanthine. In addition,  $k$  is 10-fold larger at pH 7.4 than at 5.5. These results combined indicate that H-abstraction from C1' might not be completely stopped by the formation of quarternary salts in acidic solution as was originally proposed by Terada, instead the acidic pH may simply be serving to slow down the reaction.

This rationale would also serve to explain why the value of  $n$  after 3600 s for pH = 7.4 is not closer to "2". Since the rate of decay in absorbance is especially rapid in the initial 60 s of the reaction, it is conceivable that a true representation of the initial rate is not being observed. If the reaction is sufficiently fast, it is possible that, during the process of mixing the solutions and transferring to the spectrophotometer, that a large portion of the reaction had already occurred and could not be monitored. Thus, the



perceived rate constant may not actually be a true representation of the “initial” rate constant.

By calculating an initial absorbance ( $A_{0,cal}$ ) according to the Beer-Lambert Law, it was possible to obtain a better idea of the absorbance at the onset of the reaction. Using this value, the calculations for  $n$  and  $k$  were repeated and results more consistent with those hypothesized were obtained. The values of  $n$  after 600 s and 3600 s calculated using this  $A_{0,cal}$ , were closer to the expected value of “2” and also the discrepancy in the values for rate constants at the two different pHs was less pronounced. The  $R^2$  values were much smaller than those obtained by using observed absorbance values, a phenomenon that adds support to the need to determine initial rate kinetics by alternative methods that will be discussed in Chapter 6.

The results presented in this section suggest that Terada’s proposal of an additional functional group for H-abstraction may be correct. However, no unambiguous evidence has been provided to (a) support their mechanism, or (b) to explain the lack of reactivity of the comparable C1 hydrogen.

It is the proposal of this author that the formation of a resonance structure indicating stabilization between N2’ and C1’ serves to lower the bond dissociation energy (BDE) of the C1’ hydrogen. When the amine is protonated, the BDE of the C1’ hydrogen is sufficiently higher to slow down the rate of reaction but not to completely stop the reaction from occurring.

Furthermore, computer molecular modeling does indicate that the C1 hydrogens

are more sterically crowded by the surrounding functional groups and hence, less accessible than the C1' hydrogens in both oxyacanthine (**2**) and cepharanthine (**5**). Although investigations are currently underway, no evidence has yet been provided to prove that H-abstraction from C1' is favored over H-abstraction from C1.

#### **4.3.2 The Role of Oxidation Products**

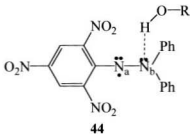
It has been the assumption that with mono-functionalized compounds, H-atom donation normally resulted in a radical species that underwent a rapid termination reaction to give products which are incapable of continuing to reduce DPPH. However, with multi-functionalized compounds such as **43**, primary oxidation products have been known to continue to quench radical species, thus forming secondary, tertiary, quaternary, etc. oxidation products. It has been reported that the primary oxidation products (phenolic radicals) can themselves quench other free radicals, an effect that may be reflected in the observed BBIQ activity and could offer an alternative explanation for the experimental values of  $n$ . Since the products of oxidation from the oxyacanthine-DPPH reaction were never isolated, further insight into the role that these compounds play in the radical reduction reaction cannot be expanded upon at this point in time.

#### **4.3.3 The Effects of Hydrogen Bonding in Radical Scavenging**

Interpretation of our results from the examination of solvent effects is essential in understanding the nature of the reaction.<sup>13,61,62,66</sup> For the purposes of this thesis, only the contributions of protic solvents, such as is found in our 2:3 Tris : EtOH buffered medium will be considered. (The effects of the buffered component is a work in progress and will

not be expanded upon in this thesis.) EtOH is a major constituent of the buffered solvent and the extent of which it H-bonds with both hydrogen-donating functional groups, as well as with radical species must be considered. Barclay *et al.*<sup>61</sup> have studied the effect of H-bonding of various solvents (propanol, *t*-butyl alcohol) on the reaction between DPPH and various antioxidants. Their findings reflect a dramatic decrease in the magnitude of second order rate constants when such solvents are used.

In addition, H-bonding also plays a role in the stability and reactivity of the radical. Valgimigli *et al.*<sup>66</sup> studied the interaction between protic solvents and the DPPH radical. As illustrated in structure **44**, the solvent can form a hydrogen bond with the lone pairs of N<sub>b</sub> on the DPPH molecule. This interaction serves to limit the de-localization of



the radical, thus making it more reactive thereby serving to speed up the reaction.

The previous two examples have demonstrated hydrogen bonding solvents as exhibiting opposite effects. Interaction with the DPPH radical results in an increase in the rate of reaction, while interaction with hydrogen-donating groups on the antioxidant serves to decrease the rate. Nonetheless, it is the additive effect of each of these contributions that must be considered when attempting to predict the magnitude of rate

constants.

#### **4.4 Summary**

In summary, rate constants and stoichiometry have been determined for oxyacanthine (**2**) and are reported in Table 6. Although there exists a discrepancy in these results, they do provide preliminary evidence that indicate the presence of a radical-scavenging functional group on the BBIQ structure that is in addition to the phenol. Also, the rate of reaction between oxyacanthine and DPPH has been demonstrated to be sensitive to pH.

## **4.5 Experimental**

### **General Section**

All analytical masses were measured on a CAHN 27 Automatic Electrobalance. All reagents and solvents used were of the highest commercial grade available from Sigma-Aldrich. Oxyacanthine sulfate was purchased from Carl Roth Pharmaceuticals (Karlsruhe, Germany), and all other solutions and/or compounds were prepared as described herein. Assays were conducted in water-incubated quartz cuvette cell holders, with absorbance values being acquired by a Varian, CARY 5E UV-Vis-NIR spectrophotometer. Absorbance was measured at  $\lambda = 516$  nm over 3,600 s unless otherwise indicated. Cuvette holders were pre-incubated for 12.0 h, and the reagent solutions were incubated for 1.0 h before use. Blank solutions were prepared by the combination of test compound solution (1.5 mL) and stock solution (1.5 mL) in the quartz cuvette followed by vortex mixing for 5 s. Analyte solutions were prepared by the combination of test compound solution (1.5 mL) and DPPH solution (1.5 mL) in the quartz cuvette followed by vortex mixing for 5 s.

### **Preparation of Reagent Solutions**

#### *PBS acetone Solutions*

Solutions of 1:1 phosphate buffered saline (PBS) media and acetone, were not stable and required preparation immediately before an assay. Quantities of DPPH and test compound were dissolved in PBS acetone stock solutions according to the concentration desired. DPPH was sonicated to aid solubility.

#### *Tris EtOH Solutions*

Stock solutions of 2:3 Tris.HCl and EtOH were adjusted to pH 7.4 and were stable for several weeks. Quantities of DPPH and test compound were dissolved in the Tris EtOH stock solutions (10 mL) according to the concentration desired. DPPH was sonicated to aid solubility.

#### *MES EtOH Solutions*

Stock solutions of 2:3 morpholine ethanesulfonic acid (MES) and EtOH were adjusted to pH 5.5 and were stable for several weeks. Quantities of DPPH and test compound were dissolved in the Tris EtOH stock solutions (10 mL) according to the concentration desired. DPPH was sonicated to aid solubility.

## CHAPTER 5

### BBIQ SYNTHESIS

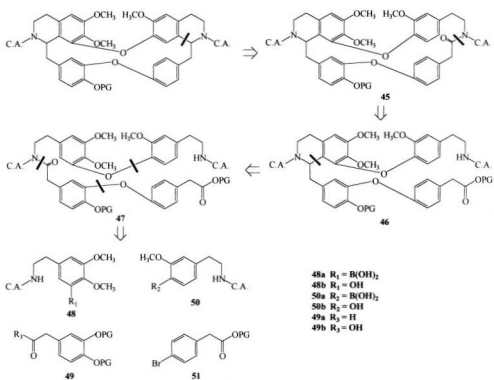
#### 5.1 Introduction

The efforts presented in this section deal with the synthesis of both the oxyacanthine (**2**) and the berbamine (**3**) types of structures. As described in Chapter 1, much of the previous success towards these targets has been attributed to the work of Kametani<sup>24,25,26</sup> and Inubushi<sup>23</sup> *et al.* Kametani proposed the coupling of two appropriately substituted halves, relying mostly upon chromatographic techniques for separation of the individual products. Inubushi examined an asymmetric route that permitted enhanced control of selectivity, allowing the construction of targeted BBIQs. Although both routes were examined in detail, only the synthetic endeavours that dealt with an asymmetric synthetic approach towards these targets will be expanded upon.

#### 5.2 Results and Discussion

##### 5.2.1 Current Proposal for the Synthesis of an Oxyacanthine-Berbamine Model

Outlined in Scheme 8 is a proposed retrosynthetic analysis for berbamine. The first retrosynthesis gives the amide (**45**) as a precursor to the Bischler-Napieralski cyclization (BNC), which should result in the formation of one product considering that there is only one site for ring closure (*para* to the electron-donating methoxy group). Structure **46** includes protected phenylacetic acid and amine moieties required for the condensation to the amide, and indicates an additional retrosynthetic cut leading to the



**Scheme 8**

amide (**47**), required for the second ring closure. The three retrosynthetic breakages shown in structure **47** reveal the four synthons represented by structures **48**, **49**, **50**, and **51**. It is at this stage that alternative methodologies may be investigated for the formation of the ether bridges and the isoquinoline rings. For this particular synthetic target, the research presented will be concentrated on the  $Cu(OAc)_2$ -mediated boronic acid coupling methodology described by Evans<sup>29</sup> and Chan.<sup>30</sup> In addition, through the endeavours needed to construct **48a**, **48b**, **49a** and **49b**, the effects of other substituents on the aromatic ring can be explored to aid in the efficiency of the coupling reaction.



The use of both the Bischler-Napieralski (requiring construction of **49b**) and the Pictet-Spengler cyclizations (requiring the construction of **49a**) would also be examined. This author's discussion will be mainly on the synthesis of compounds **48b**, **49a**, and **49b**, with particular emphasis on the potential of these synthons to contribute to ether linkage- and isoquinoline-forming reactions.

## 5.2.2 Synthesis of Two Appropriately Substituted Synthons

The synthesis of **48b** requires three oxygen functionalities on the aromatic ring and an aldehyde or acid functionality that can be further expanded into a phenylacetic acid derivative. It should be reconsidered that Cu(OAc)<sub>2</sub>-mediated boronic acid coupling methodology works best with electron-rich phenols, such as **48b**. Initial considerations proposed the route in Scheme 9, involving the bromination of vanillin (**52**) followed by derivatization and protection of the primary alcohol (**53**). This would allow halogen-

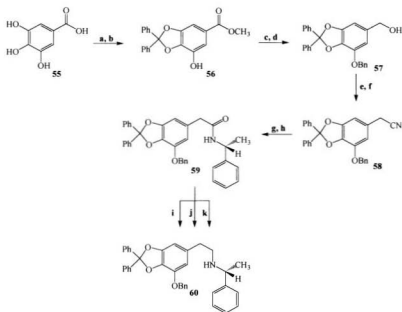


**Scheme 9**

metal exchange and work-up with hydrogen peroxide to give phenol **54**. This route was performed by Dr. You-Chu Wang and although it was successful, several reaction steps were involved, and conversion to the phenol occurred in a less than desirable yield. Thus,

a route starting with commercially-available gallic acid was explored.

Gallic acid (**55**) has three hydroxyl groups on its aromatic ring, this being a prerequisite for synthon **48b**. Of course, protection of the three phenols is necessary for convenience of aqueous work-ups, and to remove interference with some of the reaction conditions. The chosen protecting groups had to be robust enough to withstand the reaction series, but at the same time, exhibit enough versatility to be removed selectively in the presence of other protecting groups. Scheme 10 illustrates the reaction sequence, starting with the conversion of **55** to the methyl ester. Considering the symmetrical nature



**a)** MeOH, SOCl<sub>2</sub>, reflux, 96%; **b)** Cl<sub>2</sub>CPh<sub>2</sub>, 170 °C, 5 min, 79%; **c)** LiAlH<sub>4</sub>, THF, rt, 97%; **d)** acetone, K<sub>2</sub>CO<sub>3</sub>, BnBr, reflux, 92%; **e)** CH<sub>2</sub>Cl<sub>2</sub>, pyridine, SOCl<sub>2</sub>, rt, 84%; **f)** NaCN, DMSO/benzene, rt, 92%; **g)** EtOH, 4M NaOH, reflux, 85%; **h)** DCC, 4-(dimethylamino)pyridine, (*R*)-methylbenzylamine, 64%; **i)** LiAlH<sub>4</sub>, reflux, no reaction; **j)** BH<sub>3</sub>.THF, BF<sub>3</sub>.Et<sub>2</sub>O, reflux, 25%; **k)** BH<sub>3</sub>.DMS, reflux, 78%

**Scheme 10**

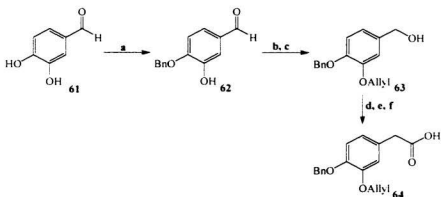
of the compound, the next step permitted protection of two of the phenolic functions with a diphenylmethane group.<sup>17</sup> Compound **56** was further reduced with  $\text{LiAlH}_4$  and the remaining hydroxyl was protected as the benzyl ether **57**. This was followed by conversion of the primary alcohol to the benzyl chloride **57a** with  $\text{SOCl}_2$  and pyridine. Initially, this reaction was performed with only  $\text{SOCl}_2$  in  $\text{CH}_2\text{Cl}_2$ , but the generation of aqueous  $\text{HCl}$  resulted in the cleavage of the diphenylmethane group. This problem was alleviated through the addition of one equivalent of pyridine, which served as an acid "mopping agent."

Cyanation gave **58**, which was followed by hydrolysis with  $\text{NaOH}/\text{EtOH}$ , to form the phenylacetic acid. Condensation with (*R*)-methylbenzylamine using DCC/DMAP afforded **59**. The introduction of a chiral auxiliary at this stage would permit for future control of stereogenic centers. The final step, involving the reduction of amide **59**, posed significant difficulty. Although literature precedence includes the successful reduction of amides with  $\text{LiAlH}_4$ ,<sup>18</sup> our efforts did not yield any significant product. Reduction with  $\text{BH}_3\cdot\text{THF}$  and a catalytic amount of  $\text{BF}_3\cdot\text{Et}_2\text{O}$  did produce some product, albeit in low yield, probably as a result of the acidic work-up serving to cleave the diphenylmethane group. Recognizing this problem, the alternative reagent  $\text{BH}_3\cdot\text{DMS}$  followed by quenching with TMEDA provided a neutral medium that did not result in cleavage of acid sensitive functionalities, and permitted the formation of the desired product **60** in 78% yield.

Scheme 10 represents an efficient route for forming the phenylacetamine **60** in

decent overall yield. The aromatic ring of **60** bears three protected oxygen substituents and a chiral amine auxilliary group, which could serve to direct stereochemistry in the isoquinoline ring closure. However, the route demonstrated extreme sensitivity to acidic conditions, a property that could hinder the success of final product formation in future reactions (*e.g.* Bischler-Napieralski cyclization). In addition, some concern was expressed over the ease with which the diphenylmethane group could be removed and replaced with the other alkyl substituents required for the final product. It should be noted that an attempt was made to cleave the diphenylmethane group and methylate the phenols. However, this sequence resulted in the formation of a black, "gummy" mixture that made work-up and subsequent purification difficult. No further attempts to isolate the product were ventured.

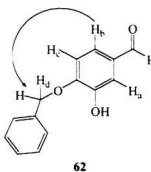
Attention was next directed towards the construction of the synthon unit **49b** (for the purpose of this particular discussion, only synthesis towards the phenylacetic acid derivative is expanded upon due to problems with the aldehyde synthesis that will be examined in section 5.2.4). Synthon **49b** required two phenolic groups to be protected with groups that could be selectively cleaved, as well as an acid or aldehyde functionality that could be easily converted to the phenylacetic acid. This compound was easily obtained from 3,4-dihydroxybenzaldehyde (**61**) according to Scheme 11. Following the method reported by Wymann *et al.*,<sup>58</sup> the phenol *para* to the electron-withdrawing aldehyde was alkylated. Under the conditions described ( $\text{Li}_2\text{CO}_3$ , DMF, 55 °C) yields from 55-90% were reported, depending on the alkylating agent and the substitution



**a**)  $\text{BnBr}$ ,  $\text{Li}_2\text{CO}_3$ , DMF,  $52^\circ\text{C}$ , 26 h, 49%; **b**) allyl bromide,  $\text{K}_2\text{CO}_3$ , acetone, reflux, 24 h; **c**)  $\text{NaBH}_4$ , 1:1 MeOH/THF, 1.5 h, 94%; **d**)  $\text{SOCl}_2$ , pyridine,  $\text{CH}_2\text{Cl}_2$ , 12.0 h, 81%; **e**)  $\text{NaCN}$ , 2:1 DMSO/benzene, 12.0 h, 91%; **f**) 2:1 EtOH: $\text{NaOH}$ , reflux, 78%

**Scheme 11**

pattern of the substrate. Repetition of the reported experiment with benzyl bromide and 3,4-dihydroxybenzaldehyde yielded the desired product **62** in 49% yield and the



**62**

dibenzylated benzaldehyde as a second major product.

Compound **62** was characterized using high-resolution NMR techniques. The HMBC indicated a correlation between what is believed to be proton  $\text{H}_b$  and the benzyl carbon, thus allowing the confirmation of structure **62**. It should be noted that  $\text{H}_b$  is indicated by COSY to be located in the middle of a multiplet of aromatic proton

peaks. Because of this uncertainty in assignment a replicate reaction was conducted using

allyl bromide as the alkylating agent. The same analysis was repeated, thus confirming substitution of the *para* phenol. If the alkyl group were protecting the *meta* hydroxy, the correlation indicated in compound **62** would not be present due to the fact that a five-bond correlation would not be likely.

The remainder of the synthetic route outlined in Scheme 11 progressed in generally high yield. The *meta* hydroxyl in **62** was protected with an allyl group, followed by reduction of the aldehyde with NaBH<sub>4</sub> resulting in compound **63**. The primary alcohol was converted to the nitrile and subsequent hydrolysis gave the phenylacetic acid **64** in good yield.

With the completion of the two “quarters” of the oxyacanthine-berbamine skeleton, the ether linkage and isoquinoline ring closure methodology could be further explored. However, at this juncture attention was diverted towards the the total synthesis of an isochondodendrine type of alkaloid, cycleanine (**10**). This permitted the synthesis of a more suitable model for DPPH radical scavenging studies. Further exploration into the synthesis of the oxyacanthine-berbamine structures stopped, but some of the products previously outlined were utilized in the construction of the new target alkaloid.

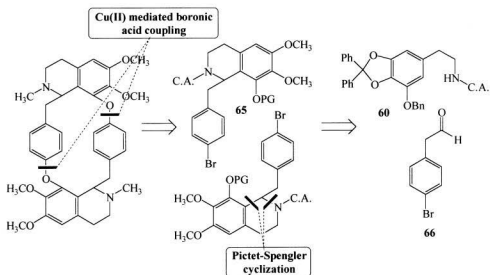
### **5.2.3 Attempts Towards the Total Synthesis of Cycleanine**

The synthesis of cycleanine **10** was not an original target. This particular compound was deemed to be useful for further studies into DPPH radical-scavenging activities of BBIQs. Cycleanine is a symmetrical head-to-tail dimer with no other radical-quenching functionalities besides the two isoquinoline rings. With this compound in

hand, future researchers may be able to determine the role of the isoquinoline stereogenic centers towards the reductive activity of DPPH. In addition, structure-activity investigations may allow for a deeper insight into other more complicated BBIQs (*e.g.* berbamine-oxyacanthine).

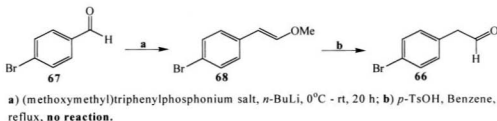
#### 5.2.4 Construction of a Tri-*axo* Substituted Benzyltetrahydroisoquinoline

Efforts were directed towards the synthesis of an appropriately-substituted benzyltetrahydroisoquinoline monomer unit as described by Tomita *et al.* (outlined in Chapter 1). Although the final step of their synthesis failed to yield the target molecule by the Ullmann coupling methodology, the use of the  $\text{Cu}(\text{OAc})_2$ -mediated boronic acid coupling, as reported by Evans and Chan was proposed to be a superior method to permit



Scheme 12

an effective dimerization. Optimized conditions for this particular reaction require a highly activated phenol to couple with a halide, a prerequisite that is obeyed and outlined in the retrosynthetic analysis of Scheme 12. The first two retrosynthetic cuts correspond to the formation of the ether bridges through coupling of monomer **65**. Compound **65** can be further derived from the combination of compounds **60** and **66** via a Pictet-Spengler cyclization. Of course, this represented a favoured route considering that the construction of **60** was already complete, thus requiring only the synthesis of the aldehyde derivative (**66**). Scheme 12 effectively outlines the necessary prerequisites for an effective ether linkage. Conversion of the bromine to a boronic acid, followed by deprotection of the phenol, should allow for the formation of the diaryl ether linkage. In addition, the Pictet-Spengler cyclization strategy would permit a milder isoquinoline formation allowing for retention of the diphenylmethane group.

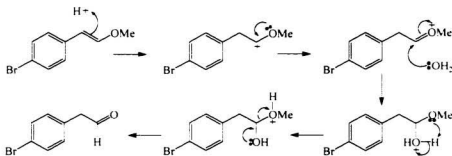


**Scheme 13**

With compound **56** in hand, attention shifted to the derivatization of the appropriately substituted aldehyde **66**. The first attempt was based on a method reported by Corey *et al.*<sup>69</sup> in their total synthesis of helminthosporal. Outlined in Scheme 13, the



Wittig reaction of 4-bromobenzaldehyde **67** with methoxymethylene triphenylphosphorane resulted in the formation of vinyl ether **68**. This was then subjected to acidic conditions in refluxing benzene to effect the transformation to aldehyde **66**. This reaction sequence was not successful and encountered a few problems, the first of which was the volatility of compound **68**. This was evidenced by the constant loss of mass of the crude product, as well as by the rapid disappearance of the product spot on TLC plates. Also, the formation of several other unidentified products made separation by column

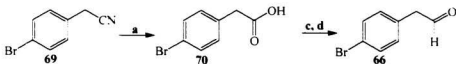


**Scheme 14**

chromatography very difficult. However, some fractions containing a mixture of *cis* and *trans* isomers were isolated and immediately subjected to the second step outlined in Scheme 13. Unfortunately, this step did not meet with any success. The reason for this was initially attributed to the stability of the phenyl-conjugate vinyl ether. However, upon subsequent examination of the reaction conditions which were employed, it was concluded that a proton source was required for the desired transformation to the aldehyde

(Scheme 14). Corey *et al.* did not convert to the aldehyde itself, but instead transformed their methyl ether into an acetal by *in-situ* reaction with ethylene glycol. The glycol served as the proton source necessary to complete the reaction. However, whether the required modification would have been successful is open to question, since the reaction was not repeated and a different strategy was employed.

Attention was shifted to alternative methods of making the phenylacetaldehyde.



**a)** 2:1 EtOH/NaOH (4M), Reflux, 12.0 h, 94%; **c)** BH<sub>3</sub>, THF, THF, reflux, 88%;  
**d)** PCC, CH<sub>2</sub>Cl<sub>2</sub>, 50%, **mixture of products**

**Scheme 15**

with emphasis being placed on the use of 4-bromophenylacetonitrile **69** as the starting compound (Scheme 15). Initial attempts involved hydrolysis of **69** to the corresponding phenylacetic acid **70** followed by reduction to the alcohol. Oxidation of the alcohol to the aldehyde proved to be surprisingly difficult. Attempts with PCC, Dess-Martin reagent, and the Swern oxidation gave the same result, a complex mixture that was very difficult to separate by conventional purification techniques. 4-Bromobenzoic acid was formed as a byproduct in each of the oxidation procedures.

Consideration was next directed towards formation of an amide from the condensation of **56** with 4-bromophenylacetic acid, thus allowing for a subsequent BNC

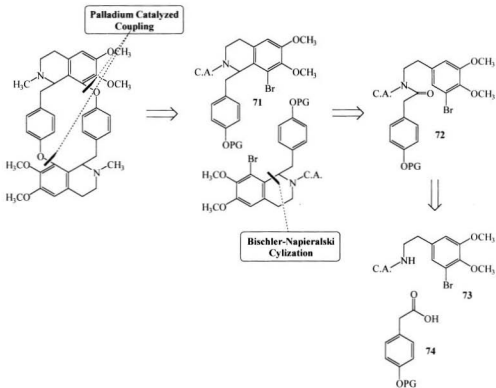
to be tried. However, this idea was rejected owing to several foreseeable difficulties, including:

- (1) The diphenylmethane group would probably not have survived the acidic environments of the BNC and subsequent reduction step.
- (2) Model studies suggested that cleavage of the diphenylmethane group and re-alkylation of the phenols would be difficult.
- (3) The presence of three oxygen functionalities on the aromatic ring could result in a mixture of regioisomers being formed in the BNC.

It was decided that these anticipated problems were significant enough to warrant temporary abandonment and re-evaluation of the synthetic route.

#### 5.2.5 Construction of Chiral Bromoarmepavine

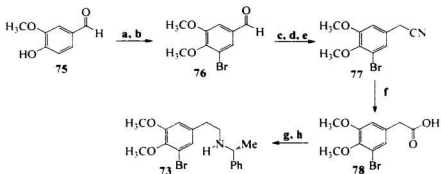
As shown in Scheme 4, although Tomita *et al.* synthesized *d,l*-bromoarmepavine (**30**), their attempts to effect a dual Ullman ether coupling on **30** to form cycleanine directly, failed. We reasoned that in our hands, this same objective might be achieved using Buchwald's<sup>31</sup> recently described methodology. Since there is no reported precedence for the coupling of an electron-rich halide with an *ortho*-substituted electron-donating group, this was an anticipated difficulty. The retrosynthetic analysis is shown in Scheme 16 which also envisioned control of the absolute stereochemistry at C1 of the tetrahydroisoquinoline ring. This could be achieved by the use of a suitable chiral auxiliary on the amine precursor which could be subjected to a Bischler-Napieralski cyclization reaction.



**Scheme 16**

Buchwald's methodology requires monomer **71** with both a benzyl phenol and an activated halide on the tetrahydroisoquinoline. Monomeric unit **71** might be derived by BNC of **72**, which in turn, would be obtained by the condensation of amine **73** and phenylacetic acid **74**.

The synthesis of the chiral amine **73** was achieved by the sequence in Scheme 17. Starting with vanillin (**75**), bromination occurred selectively at C-5, and methylation of the phenol gave compound **76**. With subsequent reduction of the aldehyde followed by derivitization to the nitrile, **77** was formed in good yield. Hydrolysis gave the

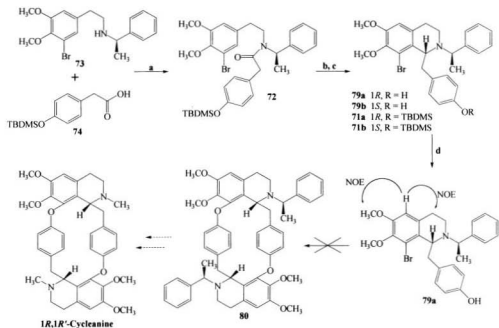


**a)** Br<sub>2</sub>, AcOH, 82%; **b)** (CH<sub>3</sub>)<sub>2</sub>SO<sub>4</sub>, NaOH, Adogen, CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O, 81%; **c)** NaBH<sub>4</sub>, MeOH/THF, 99%; **d)** SOCl<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 60%; **e)** NaCN, DMSO/Benzene, 95%; **f)** 2:1 EtOH/NaOH (4M), reflux, 95%; **g)** (*R*)-methylbenzylamine, DCC, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 88%; **h)** BH<sub>3</sub>.THF, reflux, (not purified)

#### Scheme 17

phenylacetic acid **78**. Introduction of the chiral amine through a DCC-aided condensation and reduction of the resultant amide gave the required amine synthon **73**. This route gave the desired structure with the necessary substitution pattern that would appear in the final product. In addition, many of the problems associated with acid sensitivity, and functional group modification found in the previously reported routes were circumvented.

Synthesis of the protected synthon **74** proved to be very simple. Starting with the commercially available 4-hydroxyphenylacetic acid, reaction with TBDMSCl protected the phenol and at the same time formed the TBDMS ester. The ester functionality was labile on silica gel, thus purification using silica gel column chromatography gave **74** as the sole product in 80% yield.



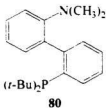
a) DCC, DMAP,  $\text{CH}_2\text{Cl}_2$ , rt, 84%; b)  $\text{POCl}_3$ , benzene, reflux; c)  $\text{NaBH}_4$ ,  $\text{MeOH/THF}$ ,  $-78^\circ\text{C}$ ; d) TBAF, THF, 30%, 3 steps

**Scheme 18**

As shown in Scheme 18, amide **72** was formed by the condensation of amine **73** and phenylacetic acid **74**. BNC and  $\text{NaBH}_4$  reduction gave a complex mixture of compounds. This mixture contained both protected and unprotected phenols (**79a,b** & **71a,b**), which could be simplified by fully deprotecting the TBDMS group with *tert*-butylammonium fluoride. Purification by preparative layer chromatography yielded the desired product (**79a**) in only 30% yield. Due to the small amount of material (18 mg), other regioisomers and diastereomers were not identified. This was a very disappointing result, considering that model studies of the BNC of amide **72** with a more robust group

(e.g. benzyl) protecting the phenol, gave the desired regioisomer and diastereomer in 70% yield and in 95% d.e.

Protecting the phenolic group was only necessary for the initial step involving the condensation of the amine and the phenylacetic acid. It was originally envisioned that the acidic medium of the BNC and aqueous work-up following the reduction step, would be sufficient to cleave completely the phenol protecting group. This in turn would have permitted two events to occur in the same operation. However, this was not the case. The newly formed zwitterionic species (**79a** & **79b**) would require the tight control of pH in the aqueous work-up, since an acidic medium would result in the formation of a quaternary ammonium salt, while a basic medium would result in the formation of a salt from the phenol. Considering that either medium risks loss of product, attempts to quench the reaction with a weak base such as TMEDA followed by subsequent aqueous work-up from water was proposed to be an effective method. However, this only served to remove the first of the problems associated with the work-up of this reaction. Both methods whether involving the quenching of the reaction with acid or TMEDA, resulted



in the formation of a stable emulsion upon the attempted aqueous extraction. This emulsion proved difficult to break, thus making extraction with organic solvents extremely difficult.

Though the yield of **79a** was disappointing, the sequence did allow the isolation of a small amount of pure product. This permitted a preliminary attempt towards the synthesis of cycleanine. The procedure of

Buchwald *et al.*, involving a catalytic amount of biphenyl ligand **80**, Pd(OAc)<sub>2</sub>, and monobasic potassium phosphate in toluene, returned only starting material and an inseparable mixture of compounds near the baseline. This result was disappointing, but as discussed previously, no examples of effective ether formation using aryl halides with *ortho*-substituted electron-donating groups have been reported using the methodology described. In addition, there are several variables in Buchwald's methodology including the choice of ligand, source of palladium (II), and the choice of base and solvent which could be varied in attempts to make the target molecule. It can be concluded that the conditions needed to effect the cyclization need to be determined.

### 5.3 Summary

Although the goal of completing the total synthesis of a bisbenzylisoquinoline was not achieved, the information presented in this thesis does offer significant promise for future endeavours. The difficulties that have been identified in both the synthesis of the oxyacanthine-berbamine and cycleanine types of structures have been noted. Such information will lead to the modification of existing synthetic proposals, thus providing a framework for future success.

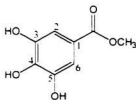


## 5.4 Experimental

### General

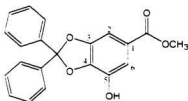
Chromatography was performed using 60 mesh silica gel and preparative layer (1mm) chromatography (PLC) with standard thin layer chromatography (TLC) grade silica gel. Flash chromatography was conducted using 230–400 mesh silica gel. All solvents and reagents used were either of the highest commercial grade available from Sigma-Aldrich and/or were redistilled ( $\text{CH}_2\text{Cl}_2$ , hexane, and benzene distilled over  $\text{CaH}_2$ ,  $\text{CHCl}_3$  distilled over  $\text{P}_2\text{O}_5$ ).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were obtained on a General Electric GE-300 NB spectrometer at 300 MHz and 75 MHz, respectively, in  $\text{CDCl}_3$  unless otherwise specified.  $^1\text{H}$  shifts are relative to an internal trimethylsilane signal, and shifts in the  $^{13}\text{C}$  spectra are relative to the solvent. Some NMR data were obtained on the Bruker Avance 500 MHz spectrometer with a TXI inverse-detect gradient probe and are indicated where applicable in the experimental. The following abbreviations are used in the description of the  $^1\text{H}$  NMR spectra: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), b (broad). NMR free induction decay (FID) data were processed using WinNuts (Acorn NMR software). Low resolution mass spectral data were recorded on the V.G. Micromass 7070HS instrument. High resolution mass spectral data were obtained from the University of Ottawa mass spectrometry centre. Melting points were determined using a Fisher-Johns hot stage apparatus and are uncorrected.

### Methyl gallate (**55a**)



To a solution of gallic acid (12.0 g, 70.5 mmol) in MeOH (200 mL) was added  $\text{SOCl}_2$  (5.63 mL, 77.6 mmol), dropwise at 0 °C. The solution was allowed to warm to room temperature before it was heated at reflux for 2.0 h. The reddish-brown solution was cooled to room temperature and evaporated on a rotary evaporator to give an off-white solid. The solid was redissolved in MeOH (100 mL portions) and concentrated *in vacuo* four times, followed by drying under high vacuum to give **55a** as a colourless solid (12.5 g, 96%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.05 (s, 2H, H-2, H-6), 3.81 (s, 3H,  $\text{OCH}_3$ ).

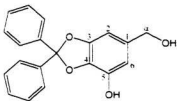
### Methyl 3,4-(diphenylmethylenedioxy)-5-hydroxybenzoate (**56**)



To methyl gallate (5.0 g, 27 mmol) was added dichloro-diphenylmethane (4.4 mL, 30 mmol). The mixture was heated to 175 °C in an oil bath for five minutes. It was then cooled to room temperature resulting in a hard black solid. This crude product was dissolved in benzene which after standing produced an off-white precipitate that was further purified by silica column chromatography (30% EtOAc:hexane) to yield **56** as a colourless solid (7.5 g, 79%); mp 165-166 °C;  $^1\text{H}$  NMR ( $\text{MeOH}-d_4$ ):  $\delta$  7.47 - 7.67 (m, 10H, Ar-H), 7.29 (d,  $J=1.5$  Hz, 1H, H-2), 7.18 (d,  $J=1.5$  Hz, 1H, H-6), 3.82 (s, 3H,  $\text{OCH}_3$ );  $^{13}\text{C}$  NMR ( $\text{MeOH}-d_4$ ):  $\delta$  168.19, 149.80, 142.02, 141.42, 139.73, 130.49, 129.49, 127.41, 125.53, 121.88, 115.09.

110.43, 103.08, 52.68; MS ( $m/z$ , intensity (%)): 348 ( $M^+$ , 53), 317 (14), 271 (100), 239 (3), 211 (10), 165 (49), 105 (76), 77 (40), 28 (25).

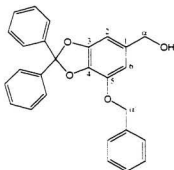
#### 5-Hydroxy-3,4-(diphenylmethylenedioxy)benzyl alcohol (**56a**)



To a suspension of  $\text{LiAlH}_4$  (85.0 mg, 2.25 mmol) in THF (15 mL) was added a solution of **56** (500 mg, 1.50 mmol) in THF (15 mL). The solution was stirred under an argon atmosphere for 4.0 h. The solvent was evaporated on a rotary evaporator. The resulting grey solid was dissolved in EtOAc (5 mL) and added to aqueous 10% HCl (5 mL) in a separatory funnel. The aqueous layer was extracted with EtOAc (3 x 5 mL), and the combined organic extracts were washed with brine (3 x 10 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent removed *in vacuo*. The resultant oil was purified by silica column chromatography (30% EtOAc hexane) to yield **56a** as a colourless solid (0.445 g, 97%); mp 160-162 °C;  $^1\text{H}$  NMR (500 MHz,  $\text{MeOH}-d_4$ ):  $\delta$  7.59-7.32 (m, 10H, Ar-H), 6.46 (s, 1H, H-2), 6.44 (s, 1H, H-6), 4.43 (s, 2H, H-a);  $^{13}\text{C}$  NMR (500 MHz,  $\text{MeOH}-d_4$ ):  $\delta$  149.93, 142.08, 141.90, 137.45, 134.70, 130.19, 129.34, 118.03, 111.21, 100.98, 65.31; MS ( $m/z$  intensity (%)): 320 ( $M^+$ , 52), 243 (93), 165 (51), 105 (100), 77 (34), 28 (26).

#### 5-Benzyloxy-3,4-(diphenylmethylenedioxy)benzyl alcohol (**57**)

To a flask charged with **56a** (200 mg, 0.66 mmol) and  $\text{K}_2\text{CO}_3$  (227 mg, 1.64 mmol) was added acetone (10 mL). The slurry was refluxed for 20 min followed by the addition of

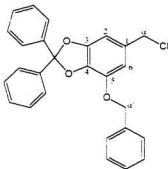


benzyl bromide (124 mg, 0.72 mmol) and was refluxed for an additional 5.0 h. The reaction mixture was allowed to cool to room temperature, filtered and concentrated using a rotary evaporator. The residue was dissolved in EtOAc (10 mL) and transferred to a separatory funnel containing H<sub>2</sub>O (10 mL). The aqueous layer was re-extracted with EtOAc (3 x 10

mL), with the combined organic portions being washed with brine (3 x 15 mL), dried over MgSO<sub>4</sub> and the solvent removed *in vacuo*. Purification by silica column chromatography (35 % EtOAc hexane) gave **57** as a colourless solid (246 mg, 92%); mp 100-101 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.58-7.34 (m, 15H, Ar-H), 6.59 (d, *J*=1.2 Hz, 1H, H-2), 6.56 (d, *J*=1.2 Hz, 1H, H-6), 5.23 (s, 2H, H-α'), 4.49 (d, *J*=4.8 Hz, 2H, H-α); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 148.74, 142.30, 140.01, 136.95, 135.31, 134.92, 129.09, 128.49, 128.18, 127.95, 127.50, 126.40, 120.27, 109.31, 101.71, 71.66, 65.34; HRMS calcd for C<sub>25</sub>H<sub>21</sub>O<sub>4</sub> 410.1517 found 410.1521

#### 5-Benzyloxy-3,4-(diphenylmethylenedioxy)benzyl chloride (**57a**)

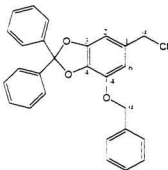
To a solution of **57** (100 mg, 0.24 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) at 0 °C was added pyridine (0.02 mL, 0.27 mmol) and SOCl<sub>2</sub> (0.02 mL, 0.27 mmol). The mixture was stirred for 1.0 h at room temperature, at which point it was transferred to a separatory funnel containing H<sub>2</sub>O (10 mL). The organic layer was separated and the aqueous layer was neutralized by the dropwise addition of aqueous 2.5% NaOH. This layer was further extracted with



$\text{CH}_2\text{Cl}_2$  (3 x 10 mL) dried over  $\text{MgSO}_4$  and concentrated in vacuo to give **57a** as a colourless oil (88.0 mg, 84%) without further purification:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.57–7.31 (m, 15H, Ar-H), 6.62 (d,  $J=1.2$  Hz, 1H, H-2), 6.58 (d,  $J=1.2$  Hz, 1H, H-6), 5.23 (s, 2H, H- $\alpha'$ ), 4.44 (s, 2H, H- $\alpha$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  148.73, 142.19, 139.86, 136.77, 131.51, 129.16, 128.52,

128.21, 128.04, 127.53, 126.36, 120.34, 111.15, 103.20, 71.77, 46.69; MS ( $m/z$ ) intensity ( $^{\circ}\text{e}$ ): 428 ( $\text{M}^+$ , 22), 393 (10), 339 (13), 337 (34), 165 (21), 157 (12), 155 (35), 105 (30), 99 (24), 92 (11), 91 (100), 77 (15), 65 (11), 28 (15).

#### 5-Benzoyloxy-3,4-(diphenylmethylenedioxy)phenylacetonitrile (**58**)

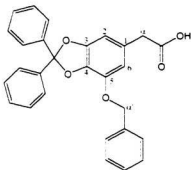


To a solution of **57a** (200 mg, 0.47 mmol) dissolved in DMSO (5 mL) and benzene (2.5 mL) was added NaCN (57.2 mg, 1.17 mmol) batchwise at room temperature. After approximately 1.0 h the solution turned cloudy. The reaction was continued for 22.0 h after which it was transferred to a separatory funnel containing  $\text{H}_2\text{O}$  (10 mL). An emulsion formed and

brine was added to stabilize and allow extraction of the aqueous layer with EtOAc (3 x 10 mL). The combined organic extracts were washed with brine (3 x 10 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent evaporated on a rotary evaporator. The resultant product

was purified via preparative layer chromatography (30% EtOAc/hexane) to yield **58** as a colourless oil (182 mg, 92 %):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.57-7.31 (m, 15H, Ar-H), 6.53 (d,  $J=1.2$  Hz, 1H, H-2), 6.51 (d,  $J=1.2$  Hz, 1H, H-6), 5.24 (s, 2H, H- $\alpha'$ ), 3.58 (s, 2H, H- $\alpha$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  190.95, 149.13, 142.49, 139.71, 136.61, 135.20, 129.23, 128.56, 128.25, 128.10, 127.54, 126.33, 117.82, 110.39, 102.51, 71.83, 23.43; MS ( $m/z$ ) intensity (%): 419 ( $M^+$ , 15), 328 (27), 165 (10), 146 (30), 105 (15), 91 (100), 90 (20).

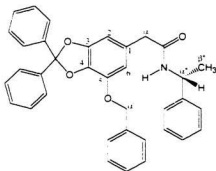
#### 5-Benzoyloxy-3,4-(diphenylmethylenedioxy)phenylacetic acid (**58a**)



A solution of **58** (536 mg, 1.30 mmol) in EtOH (10 mL) and aqueous 4.0 M NaOH (5 mL) was refluxed for 20 h. The reaction was allowed to cool to room temperature and was added to  $\text{H}_2\text{O}$  (10 mL) in a separatory funnel. The aqueous layer was acidified with aqueous concentrated HCl and extracted with  $\text{CH}_2\text{Cl}_2$  (3 x 10 mL). The combined organic extracts were washed with brine (3 x 10 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent was evaporated on a rotary evaporator to give an off-white solid. The product was recrystallized from EtOAc/hexane to give colourless crystals (487 mg, 85 %), mp 141-142  $^\circ\text{C}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.57-7.29 (m, 15H, Ar-H), 6.52 (d,  $J=1.5$  Hz, 1H, H-2), 6.47 (d,  $J=1.5$  Hz, 1H, H-6), 5.22 (s, 2H, H- $\alpha'$ ), 3.47 (s, 2H, H- $\alpha$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  177.56, 148.69, 142.23, 140.03, 136.88, 134.69, 129.07, 128.48, 128.17, 127.96, 127.56, 127.04, 126.39, 120.26, 111.55, 103.81, 71.72, 40.79; MS ( $m/z$ ) intensity

( $^{\circ}$ ): 438 (M<sup>+</sup>, 36), 347 (54), 165 (42), 137 (16), 105 (38), 91 (100), 67 (23); HRMS calcd for C<sub>28</sub>H<sub>21</sub>O<sub>4</sub> 438.1466 found 438.1483

***N*-[(*R*)-Methylbenzyl]-(5-benzyloxy-3,4-diphenylmethylenedioxy)phenylacetamide (**59**)**



To a solution of **58a** (219 mg, 1.10 mmol),

(*R*)-methylbenzylamine (66 mg, 0.5 mmol),

and 4-(dimethylamino) pyridine (10 mg) in

CH<sub>2</sub>Cl<sub>2</sub> (2.5 mL) was added DCC (103 mg,

1.00 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (2.5 mL).

After approximately 1.0 h a white precipitate

had formed, the reaction mixture was stirred

for an additional 45 h under an argon atmosphere. The reaction mixture was filtered

through a plug of glass wool in a pasteur pipette, followed by washings with aqueous

saturated NaHCO<sub>3</sub> (3 x 5 mL). The organic layer was dried over MgSO<sub>4</sub>, filtered and the

solvent removed in vacuo. Purification by silica column chromatography (50%

EtOAc/hexane), yielded **59** as a colourless solid (188 mg, 64%); mp 130-131°C; <sup>1</sup>H NMR

(500 MHz, CDCl<sub>3</sub>): δ 7.58-7.16 (m, 20H, Ar-H), 6.49 (s, 1H, H-2), 6.43 (s, 1H, H-6), 5.58

(d, *J*=7.6 Hz, 1H, N-H), 5.22 (s, 2H, H-α'), 5.09 (m, 1H, H-α), 3.43 (s, 2H, H-α''), 1.37 (d,

*J*=7.0 Hz, 3H, H-β''); <sup>13</sup>C NMR (500 MHz, CDCl<sub>3</sub>): δ 169.88, 149.06, 143.05, 142.51,

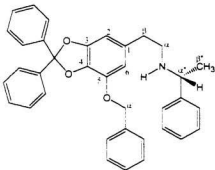
140.03, 136.79, 134.80, 129.14, 128.85, 128.58, 128.52, 128.24, 128.03, 127.49, 127.23,

126.35, 125.92, 117.50, 111.63, 103.81, 71.75, 48.68, 43.76, 21.76; MS (*m/z*) intensity

( $^{\circ}$ ): 541 (M<sup>+</sup>, 20), 393 (11), 106 (11), 105 (100); HRMS calcd for C<sub>36</sub>H<sub>31</sub>NO<sub>4</sub> 541.2251

found 541.2268

**N-[(*R*)-Methylbenzyl]-(5-benzyloxy-3,4-diphenylmethylenedioxy)phenylacetamine (60)**



Compound **59** (59 mg, 0.1 mmol) was dissolved in THF (3 mL) and brought to a gentle reflux for 15 min. To this solution was added  $\text{BH}_3 \cdot \text{DMS}$  (24 mg, 0.2 mmol) dropwise. After 50 min the reaction was cooled to room temperature. TMEDA (18 mg, 0.2 mmol) was added and the solution was

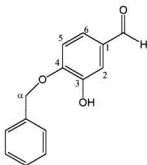
stirred for an additional 20.5 h. The solvent was evaporated on a rotary evaporator and yielded a solid which was purified via preparative layer chromatography (5% MeOH  $\text{CHCl}_3$ ).

Compound **60** was obtained as a colourless oil (45 mg, 78%).  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  7.57-7.20 (m, 20H, Ar-H), 6.38 (s, 1H, H-2), 6.34 (s, 1H, H-6), 5.20 (s, 2H, H- $\alpha'$ ), 3.71 (q,  $J=6.5$  Hz, 1H, H- $\alpha''$ ), 2.60 (m, 5H, H- $\alpha$ , H-2, N-H), 1.29 (d,  $J=6.6$  Hz, 3H, H- $\beta''$ )

**4-Benzyloxy-3-hydroxybenzaldehyde (62)**

To a solution of 3,4-dihydroxybenzaldehyde (500 mg, 3.60 mmol) in DMF (15 mL) was added  $\text{Li}_2\text{CO}_3$  (665 mg, 9.00 mmol), and benzyl bromide (681 mg, 4.00 mmol). The yellow slurry was stirred under an argon atmosphere at 52 °C for 26 h. The mixture was transferred to a separatory funnel containing  $\text{H}_2\text{O}$  (250 mL) and aqueous 10% HCl (5 mL). The aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (3 x 20 mL), with the combined

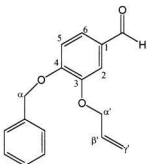




organic extracts being dried over  $\text{MgSO}_4$ , filtered and the solvent evaporated on a rotary evaporator. Purification by silica column chromatography (40% EtOAc/hexane) yielded the desired product (**62**) as a yellow solid (0.400 g, 49%), mp 119 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  9.82 (s, 1H, -COH), 7.45-7.39 (m, 7H, Ar-H), 7.04 (d,  $J=5.0$ , 1H, H-2), 5.90 (s, 1H,

Ar-OH), 5.20 (s, 2H, H- $\alpha$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  191.24, 151.17, 146.55, 135.46, 131.05, 129.11, 129.02, 128.13, 124.56, 114.65, 111.75, 71.51; MS ( $m/z$ ) intensity (%): 228 ( $\text{M}^+$ , 1) 91 (100), 65 (12).

### 3-Allyloxy-4-benzyloxybenzaldehyde (**62a**)

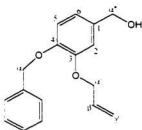


A slurry of **62** (200 mg, 0.88 mmol) and  $\text{K}_2\text{CO}_3$  (300 mg, 2.20 mmol) in acetone (12 mL) was brought to a gentle reflux. After approximately 20 min, allyl bromide (117 mg, 0.97 mmol) was added dropwise and the resultant mixture was refluxed for an additional 5.0 h.

The solution was filtered and the solvent evaporated on a rotary evaporator to give a yellow solid. The solid was redissolved in EtOAc (20 mL) and transferred to a separatory funnel containing  $\text{H}_2\text{O}$  (20 mL). The aqueous layer was extracted with EtOAc (3 x 15 mL), and the combined organic layers were washed with brine (3 x 10 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent evaporated on a rotary evaporator to give a yellow oil. Purification via preparative layer chromatography (30%

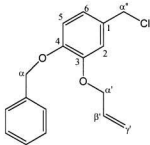
EtOAc/hexane) gave **62a** as a colourless solid (201 mg, 85%), mp 37 °C: <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 9.82 (s, 1H, -COH), 7.46-7.30 (m, 7H, Ar-H), 7.01 (d, *J*=6.0 Hz, 1H, H-2), 6.09 (m, 1H, H-β'), 5.49-5.42 (2 x q, *J*=1.5 Hz, 1H, H-α'), 5.34-5.29 (2 x q, *J*=1.4 Hz, 1H, H-α'), 5.25 (s, 2H, H-α), 4.70-4.67 (2 x t, *J*<sub>AB</sub>=5.2 Hz, *J*<sub>AX</sub>=1.5 Hz, 2H, H-γ').

### 3-Allyloxy-4-benzoyloxybenzyl alcohol (**63**)



To a solution of **62a** (177 mg, 0.66 mmol), in MeOH (10 mL) and THF (10 mL) was added NaBH<sub>4</sub> (15 mg, 0.3 mmol) batchwise at room temperature. The mixture was stirred for 1.5 h following which, the solvent was removed *in vacuo*. The grey solid was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) and quenched with aqueous 10% HCl (10 mL). The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 10 mL) and the combined organic layers were washed with brine (3 x 20 mL), dried over MgSO<sub>4</sub> filtered and the solvent evaporated on a rotary evaporator to give a colourless oil. Purification by PLC (30% EtOAc/hexane) resulted in **63** (123 mg, 70%) as a colourless oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.44-6.79 (m, 8H, Ar-H), 6.08 (m, 1H, H-β'), 5.45-5.39 (2 x q, *J*=1.5 Hz, 1H, H-α'), 5.34-5.29 (2 x q, *J*=1.4 Hz, 1H, H-α'), 5.25 (s, 2H, H-α), 4.70-4.67 (2 x t, *J*<sub>AB</sub>=5.2 Hz, *J*<sub>AX</sub>=1.5 Hz, 2H, H-α''); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 148.76, 148.01, 137.16, 134.19, 133.31, 128.41, 127.69, 127.12, 119.76, 117.52, 114.64, 113.22, 71.12, 69.81, 65.05; MS (*m/z*, intensity (%)): 270 (M<sup>+</sup>, 8), 91 (100), 41 (24).

### 3-Allyloxy-4-benzyloxybenzyl chloride (63a)

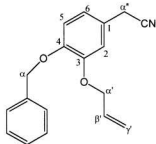


To a solution of **63** (1.5 g, 5.5 mmol) in  $\text{CH}_2\text{Cl}_2$  (75 mL) was added  $\text{SOCl}_2$  (430 mg, 6.05 mmol, 1.1 eq) dropwise at  $0^\circ\text{C}$ .

The solution was stirred at room temperature for 2.0 h at which point, it was quenched with water, neutralized with aqueous 25% NaOH and extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 75 mL).

The combined organic extracts were washed with brine (3 x 100 mL) dried over  $\text{MgSO}_4$  and the solvent evaporated on a rotary evaporator. The crude product was used in the subsequent step without further purification (1.6 g, 98%).

### 3-Allyloxy-4-benzyloxyphenylacetonitrile (63b)

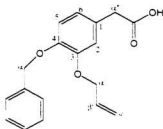


To a solution of **63a** (1.6 g, 5.4 mmol) in DMSO (35 mL) and benzene (17.5 mL) was added NaCN (658 mg, 13.4 mmol) batchwise. After stirring for 4.5 h reaction mixture was added to  $\text{H}_2\text{O}$  (200 mL) in a separatory funnel. The aqueous layer was extracted with EtOAc (3 x 75 mL),

washed with brine (3 x 100 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent evaporated on a rotary evaporator. The crude product was purified by silica column chromatography to give **63b** as a yellow oil (1.4 g, 91%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.46-6.78 (m, 8H, Ar-H), 6.08 (m, 1H, H- $\beta'$ ), 6.47-5.41 (2 x q,  $J$ =1.6 Hz, 1H, H- $\alpha'$ ), 5.32-5.29 (2 x q,  $J$ =1.4 Hz, 1H, H- $\alpha'$ ), 5.15 (s, 2H, H- $\alpha$ ), 4.64-4.62 (2 x t,  $J_{\text{AB}}$ =5.3 Hz,  $J_{\text{AX}}$ =1.5 Hz, 2H, H- $\gamma'$ ), 3.66 (s, 2H, H- $\alpha''$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  149.01, 148.27, 136.86, 132.96, 128.47, 127.81, 127.11,

122.65, 120.56, 117.85, 114.89, 113.77, 71.05, 69.94, 23.09; MS ( $m/z$ , intensity (%)): 279 ( $M^+$ , 3), 91 (100), 41 (10).

### 3-Allyloxy-4-benzyloxyphenylacetic acid (**64**)



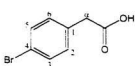
A solution of **63b** (1.15 g, 4.12 mmol) in EtOH (50 mL) and NaOH (25 mL, 4M) was refluxed for 20 h. The solution was allowed to cool to room temperature followed by addition to H<sub>2</sub>O (50 mL) in a separatory funnel. The aqueous layer was acidified with aqueous concentrated HCl and extracted with EtOAc (3 x 50

mL). The combined organic layers were washed with brine (3 x 50 mL), dried over MgSO<sub>4</sub>, filtered and the solvent evaporated on a rotary evaporator to give a yellow oil.

Compound **64** was isolated by crystallization from EtOAc/hexane as a colourless crystalline solid (0.96 g, 78%). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.45-6.74 (m, 8H, Ar-H), 6.07 (m, 1H, H-β'), 5.44-5.38 (2 x q,  $J=1.5$  Hz, 1H, H-α'), 5.28-5.25 (2 x q,  $J=1.8$  Hz, 1H, H-α'), 5.12 (s, 2H, H-α), 4.61-4.59 (2 x t,  $J_{\text{AB}}=5.4$  Hz,  $J_{\text{AX}}=1.5$  Hz, 2H, H-γ'), 3.54 (s, 2H, H-α''); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 178.01, 148.68, 147.99, 137.21, 133.30, 128.47, 127.76, 127.17, 126.26, 122.05, 117.68, 115.44, 114.69, 71.14, 69.98, 40.57; MS ( $m/z$ , intensity (%)): 298 ( $M^+$ , 7), 91 (100), 41 (26), 28 (14).

### 4-Bromophenylacetic acid (**70**)

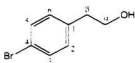
A solution of 4-bromophenylacetonitrile (1.0 g, 5.1 mmol) in EtOH (20 mL) and aqueous 4.0 M NaOH (10 mL) was refluxed for 20 h. The solution was allowed to cool to room



temperature followed by addition to H<sub>2</sub>O (25 mL) in a separatory funnel. The aqueous layer was acidified with aqueous 10% HCl and extracted with EtOAc (3 x 50 mL).

The combined organic layers were washed with brine (3 x 50 mL), dried over MgSO<sub>4</sub>, filtered and concentrated in vacuo to give **70** as an off-white solid (1.0 g, 94%). (BC **9**, 451) <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.46 (d, *J* = 8.3 Hz, 2H, H-3, H-5), 7.16 (d, *J* = 8.3 Hz, 2H, H-2, H-6), 3.61 (s, 2H, H-α); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 190.96, 177.41, 132.04, 131.75, 131.09, 121.48, 120.29, 40.34; MS (*m/z*, intensity (%)): 216 (37), 214 (M<sup>+</sup>, 35), 171 (100), 169 (96), 90 (32), 63 (14)

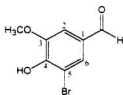
#### 2-(4-Bromophenyl)ethyl alcohol (**70a**)



Compound **70** (100 mg, 0.47 mmol) was dissolved in THF (7 mL) and refluxed for 15 min. To this solution was added BH<sub>3</sub>·THF (1.2 mL, 1.0 mol/L) dropwise and the resultant mixture was stirred at room temperature for 5.0 h. Aqueous 4.5 M HCl (5.0 mL) was added and the solution was stirred for an additional 12.0 h. The solvent was then evaporated on a rotary evaporator, with the residue being dissolved in EtOAc (5 mL) and transferred to a separatory funnel containing H<sub>2</sub>O (5 mL). The aqueous layer was basified with aqueous 4.0 M NaOH, and re-extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine (3 x 10 mL) and dried over MgSO<sub>4</sub>. Filtering of the solvent followed by evaporation on a rotary evaporator gave a colourless oil (77 mg, 83%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.43 (d, *J* = 8.4 Hz, 2H, H-3, H-5), 7.11 (d, *J* = 8.4

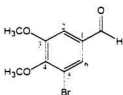
Hz, H-2, H-6), 3.84 (t,  $J = 6.5$  Hz, 2H, H- $\beta$ ), 2.82 (t,  $J = 6.5$  Hz, 2H, H- $\alpha$ ), 1.42 (s, 1H, -OH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  137.77, 131.83, 130.96, 120.54, 63.59, 38.76; MS ( $m/z$ ), intensity (%): 200 ( $M^+$ , 30), 172 (26), 169 (100), 91 (61), 90 (42), 89 (27).

### 5-Bromovanillin (75a)



To a solution of vanillin (10.0 g, 65.7 mmol) in AcOH (50 mL) was added bromine (11.5 g, 72.3 mmol) drop-wise at 0 °C. The orange suspension was stirred at room temperature for 1.0 h, at which point it was transferred to a beaker containing ice water (250 mL). The resultant solid was collected via vacuum filtration, washed repeatedly with water and air dried to give an off-white solid (12.5 g, 82%); mp 162-163 °C (Lit.<sup>14</sup>, m.p. 163-164 °C);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  9.79 (s, 1H, COH), 7.65 (d,  $J = 1.7$  Hz, 1H, H-6), 7.37 (d,  $J = 1.7$  Hz, 1H, H-2), 6.52 (s, 1H, -OH), 3.99 (s, 3H, -OCH<sub>3</sub>).

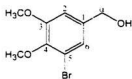
### 5-Bromo-3,4-dimethoxybenzaldehyde (76)



Compound **75a** (10.0 g, 43.3 mmol), dimethyl sulfate (8.18 g, 64.9 mmol), NaOH (4.33 g, 108 mmol),  $\text{CH}_2\text{Cl}_2$  (150 mL) and  $\text{H}_2\text{O}$  (150 mL) were combined with Adogen 464 (0.6 g). The mixture was stirred vigorously at room temperature with a mechanical stirring bar for 42.0 h. The layers were separated and the aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (3 x 100 mL), washed with brine (3 x 100 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent was removed *in vacuo* to give a yellow oil. The crude product was purified via a short silica column (35 % EtOAc/hexane) to give **76** as a colourless

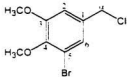
solid (8.6 g, 81%):  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  9.85 (s, 1H, COH), 3.97 (d,  $J=1.8$  Hz, 1H, H-6), 7.66 (d,  $J=1.8$  Hz, 1H, H-2), 3.95 (s, 3H,  $\text{OCH}_3$ ), 3.94 (s, 3H,  $\text{OCH}_3$ ).

#### 5-Bromo-3,4-dimethoxybenzyl alcohol (76a)



To a solution of **76** (7.92 g, 32.3 mmol) in MeOH (150 mL) and THF (150 mL) was added  $\text{NaBH}_4$  (734 mg, 19.9 mmol) batchwise over a period of 15 min. The solution was stirred at room temperature for 11.0 h followed by removal of the solvent on a rotary evaporator. The yellow solid was dissolved with aqueous 10% HCl (100 mL) and transferred to a separatory funnel. The aqueous layer was extracted with EtOAc (3 x 50 mL) and the combined organic extracts were washed with brine (3 x 50 mL), dried over  $\text{MgSO}_4$ , filtered and reduced *in vacuo* to give a yellow tinted oil. The product was used in the next step without further purification (7.98 g, 100%). (CAS 52783-74-1)  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  7.08 (d,  $J=1.5$  Hz, 1H, H-6), 7.86 (d,  $J=1.5$  Hz, 1H, H-2), 4.59 (s, 2H, H- $\alpha$ ), 3.86 (s, 3H,  $\text{OCH}_3$ ), 3.83 (s, 3H,  $\text{OCH}_3$ ).

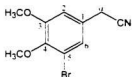
#### 5-Bromo-3,4-dimethoxybenzyl chloride (76b)



To a solution of **76a** (7.98 g, 32.3 mmol) in  $\text{CH}_2\text{Cl}_2$  (300 mL) was added  $\text{SOCl}_2$  (4.23 g, 35.5 mmol) drop-wise at  $0^\circ\text{C}$ . The solution was stirred at room temperature for 1.5 h at which point it was quenched with water, neutralized with aqueous 25% NaOH and extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 100 mL). The combined organic extracts were washed with brine (3 x 100 mL) dried over  $\text{MgSO}_4$ , filtered and the solvent evaporated on a rotary evaporator.

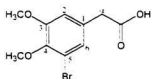
Purification by silica column chromatography (30 % EtOAc/hexane) gave **76a** as a colourless solid (4.8 g, 60%). This product was continued to the next step without further isolation or characterization.

#### 5-Bromo-3,4-dimethoxyphenylacetonitrile (**77**)



To a solution of **76b** (4.80 g, 18.1 mmol) in DMSO (100 mL) and benzene (50 mL) was added NaCN (2.20 g, 45.2 mmol) batchwise. The mixture was stirred for 12 h at room temperature. The reaction was quenched by addition to H<sub>2</sub>O (200 mL) in a separatory funnel. The aqueous layer was extracted with EtOAc (3 x 75 mL), washed with brine (3 x 100 mL), dried over MgSO<sub>4</sub> and the solvent removed *in vacuo* to give **77** as a colourless oil (4.42 g, 95%). The product was continued to the next step without further characterization.

#### 5-Bromo-3,4-dimethoxyphenylacetic acid (**78**)

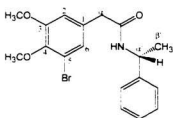


A solution of **77** (4.42 g, 16.1 mmol) in EtOH (50 mL) and aqueous 4.0 M NaOH (25 mL) was refluxed for 20 h. The solution was allowed to cool to room temperature followed by addition to H<sub>2</sub>O (50 mL) in a separatory funnel. The aqueous layer was acidified with aqueous concentrated HCl and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with brine (3 x 50 mL), dried over MgSO<sub>4</sub>, filtered and the solvent evaporated on a rotary evaporator to give a yellow oil. Compound **78** was isolated by crystallization from EtOAc/hexane as a



yellow crystalline product (2.48 g, 52%); mp 94-95 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.07 (d,  $J=1.7$  Hz, 1H, H-6), 6.79 (d,  $J=1.7$  Hz, 1H, H-2), 3.87 (s, 3H,  $\text{OCH}_3$ ), 3.85 (s, 3H,  $\text{OCH}_3$ ), 3.58 (s, 2H, H- $\alpha$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  177.22, 153.59, 145.73, 130.10, 125.46, 117.63, 112.79, 60.57, 56.08, 40.37; MS ( $m/z$ , intensity (%)): 274 ( $M^+$ , 100), 261 (23), 231 (93), 229 (93), 185 (17), 108 (20), 77 (33).

***N*-[(*R*)-Methylbenzyl]-(5-bromo-3,4-dimethoxy)phenylacetamide (**78a**)**

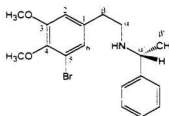


To a solution of **78** (0.4 g, 1.5 mmol), 4-dimethylaminopyridine (20 mg) and (*R*)-methylbenzylamine (85 mg, 0.7 mmol) in  $\text{CH}_2\text{Cl}_2$  (4.0 mL), was added DCC (0.3 g, 1.5 mmol) dissolved in  $\text{CH}_2\text{Cl}_2$  (4.0 mL). After approximately 1.0 h a white

precipitate had formed. The reaction mixture was stirred at room temperature for an additional 4 d. The solution was filtered through a plug of glasswool in a pasteur pipette, washed with aqueous saturated  $\text{NaHCO}_3$  (3 x 5 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent evaporated on a rotary evaporator to give a yellow residue. Purification via silica column chromatography (60% EtOAc hexane) gave **78a** as a colourless solid (0.266 g, 88%); mp 133-134 °C;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.33-7.23 (m, 5H, Ar-H), 7.01 (d,  $J=1.1$  Hz, 1H, H-6), 6.75 (d,  $J=1.1$  Hz, 1H, H-2), 5.66 (d,  $J=6.4$  Hz, 1H, N-H), 5.13 (m, 1H, H- $\alpha'$ ), 3.84 (s, 3H,  $\text{OCH}_3$ ), 3.81 (s, 3H,  $\text{OCH}_3$ ), 3.47 (s, 2H, H- $\beta'$ ), 1.45 (d,  $J=7.0$  Hz, 3H, H- $\alpha'$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  169.19, 153.83, 145.68, 142.89, 131.96, 128.66, 127.40, 125.99, 125.25, 117.77, 112.51, 60.56, 56.04, 48.88, 43.14, 21.63; MS ( $m/z$ , intensity

( $^{\circ}$ ): 377 (M<sup>+</sup>, 13), 231 (60), 229 (62), 105 (100), 77 (16), 28 (27).

**N-[(*R*)-Methylbenzyl]-[5-bromo-3,4-dimethoxy]phenylethylamine (73)**

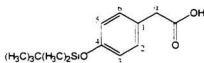


Compound **78a** (0.15 g, 0.40 mmol) was dissolved in THF (3 mL) and refluxed for 15 min. To this solution was added  $\text{BH}_3 \cdot \text{THF}$  (1.0 mL, 1.0 mol/L) dropwise.

The solution was stirred for 2.0 h. Aqueous 4.5 M HCl (3.0 mL) was added and the solution was stirred

for an additional 12.0 h. The solvent was then evaporated on a rotary evaporator, the residue dissolved in EtOAc (5 mL) and transferred to a separatory funnel containing  $\text{H}_2\text{O}$  (5 mL). The aqueous layer was basified with aqueous 4.0 M NaOH and extracted with EtOAc (3 x 5 mL). The combined organic layers were washed with brine (3 x 10 mL) and dried over  $\text{MgSO}_4$ . The solvent was filtered and evaporated on a rotary evaporator to give **73** as a yellow oil (0.13 g, 87%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.33-7.22 (m, 5H, Ar-H), 6.93 (d,  $J=1.1$  Hz, 1H, H-6), 6.64 (d,  $J=1.1$  Hz, 1H, H-2), 3.83 (s, 3H,  $\text{OCH}_3$ ), 3.82 (s, 3H,  $\text{OCH}_3$ ), 3.76 (q,  $J=6.6$  Hz, 1H, H- $\alpha'$ ), 2.78-2.64 (m, 5H, N-H, H- $\beta$ , H- $\alpha$ ), 1.33 (d,  $J=6.6$  Hz, 3H, H- $\beta'$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  153.73, 145.79, 145.00, 137.62, 128.70, 127.19, 126.77, 124.82, 117.71, 112.50, 60.82, 58.51, 56.35, 48.81, 36.31, 24.63.

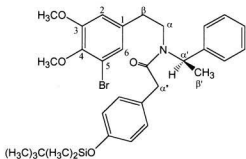
**4-(*tert*-Butyldimethylsilyloxy)phenylacetic acid (74)**



A mixture of 4-hydroxyphenylacetic acid (250 mg, 1.60 mmol), TBDMSCl (545 mg, 3.60 mmol), imidazole (280 mg, 4.10 mmol), and

DMF (2.0 mL) was combined under an argon atmosphere and stirred at room temperature for 17 h. The mixture was then added to H<sub>2</sub>O (15 mL) in a separatory funnel. The aqueous layer was extracted with EtOAc (3 x 5 mL), and the combined organic extracts were washed with aqueous saturated NH<sub>4</sub>Cl (3 x 10 mL) and dried over MgSO<sub>4</sub>. The organic solvent was filtered and evaporated on a rotary evaporator with the resultant residue being re-dissolved in MeOH (6 mL). To this solution was added silica followed by stirring at room temperature for 17 h. The MeOH was removed *in-vacuo* and the crude product was purified via column chromatography to give **74** as a colourless oil (344 mg, 80%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.13 (d, *J*=8.2 Hz, 2H, H-2, H-6), 6.78 (d, *J*=8.2 Hz, 2H, H-3, H-5), 3.56 (s, 2H, H-α), 0.97 (s, 6H, Si-CH<sub>3</sub>), 0.19 (s, 9H, C-CH<sub>3</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 178.36, 155.16, 130.56, 126.11, 120.37, 40.55, 25.89, 18.40, - 4.20.

***N*-[2-(5-Bromo-3,4-dimethoxy)phenylethyl]-*N*-[(*R*)-methylbenzyl]-4-(*tert*-butyldimethylsilyloxy)phenylacetamide (**72**)**

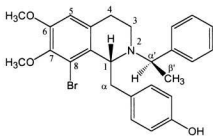


To a solution of **74** (93 mg, 0.4 mmol), 4-dimethylaminopyridine (10 mg) and **73** (64 mg, 0.2 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (2.0 mL) was added DCC (82 mg, 0.4 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (2.0 mL) under an argon atmosphere. After approximately 1.0 h a

white precipitate had formed, the reaction mixture was stirred at room temperature for an additional 4 d. At this point, the solution was filtered through a plug of glasswool in a

pasteur pipette, washed with aqueous saturated  $\text{NaHCO}_3$  (3 x 5 mL), dried over  $\text{MgSO}_4$ , filtered and concentrated *in vacuo* to give a yellow solid. Purification via silica gel column chromatography (30% EtOAc/hexane) gave **72** as a colourless oil (60 mg, 57%);  $^1\text{H}$  NMR (500 MHz, 393 K, DMSO):  $\delta$  7.35-6.66 (m, 11H, , Ar-H), 5.47 (bs, 1H, H- $\alpha'$ ), 3.80 (s, 3H,  $\text{OCH}_3$ ), 3.73 (s, 3H,  $\text{OCH}_3$ ), 3.30 (m, 2H, H- $\alpha$ , H- $\beta$ ), 2.82 (bs, 2H, H- $\alpha''$ ), 2.65 (m, 1H, H- $\alpha$ ), 2.35 (m, 1H, H- $\beta$ ), 1.51 (d,  $J = 7.1$  Hz, 3H, H- $\beta'$ ), 0.99 (s, 9H, *t*-butyl-H), 0.21 (s, 6H, Si- $\text{CH}_3$ ); MS ( $m/z$ , intensity (%): 611 ( $\text{M}^+$ , 0.4), 369 (5), 221 (28), 161 (31), 134 (45), 105 (100), 73 (40).

***N*-[(*R*)-Methylbenzyl]-8-bromo-6,7-dimethoxy-[(1*R*)-4-hydroxybenzyl]-tetrahydroisoquinoline (**79a**)**



Compound **72** (77 mg, 0.1 mmol),  $\text{POCl}_3$  (1 mL) and benzene (2 mL) were combined under an atmosphere of argon and brought to a gentle reflux. After approximately 2.0 h a dark green colour formed, the reaction was continued for an

additional 11.5 h. The solvent was evaporated on a rotary evaporator and further removed on a vacuum pump for 12.0 h. The resultant green residue was redissolved in MeOH (2 mL) and the solution was cooled to  $-78^\circ\text{C}$  in a dry ice bath. To this solution was added  $\text{NaBH}_4$ , batchwise over a period of 3.5 h. The reaction was quenched through the addition of aqueous 10% HCl (1 mL) and allowed to stir at room temperature for 15 min. The MeOH was evaporated on a rotary evaporator and the residue was redissolved in  $\text{CH}_2\text{Cl}_2$  (2 mL) and transferred to a separatory funnel containing  $\text{H}_2\text{O}$  (2 mL). The

combined aqueous and organic layers formed an emulsion that could not be discharged. The solution was brought to neutral pH by the addition of aqueous saturated  $\text{NaHCO}_3$  and the emulsion was further extracted with  $\text{CH}_2\text{Cl}_2$  (5 x 2 mL). The combined organic layers were washed with brine (3 x 5 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent evaporated on a rotary evaporator. The residue was re-dissolved in THF (3 mL), to which TBAF was added dropwise at 0 °C. The initially green solution immediately turned brown and was stirred for 2.0 h under an argon atmosphere. Upon completion of the reaction, the THF was evaporated on a rotary evaporator, the residue was dissolved in  $\text{CH}_2\text{Cl}_2$  (2 mL) and added to  $\text{H}_2\text{O}$  (2 mL) in a separatory funnel. The aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (3 x 2 mL) and the combined organic layers were washed with brine (3 x 5 mL), dried over  $\text{MgSO}_4$ , filtered and the solvent evaporated on a rotary evaporator. The resultant yellow residue was purified by preparative layer chromatography (40% EtOAc:hexane, 0.5% Et<sub>3</sub>N) yielding **79a** as a colourless oil (0.019g, 30%); <sup>1</sup>H NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.11-6.70 (m, 9H, Ar-H), 6.65 (s, 1H, H-5), 3.87 (s, 3H,  $\text{OCH}_3$ ), 3.84 (s, 3H,  $\text{OCH}_3$ ), 3.55-3.49 (m, 2H, H-3, H- $\alpha'$ ), 3.27-3.29 (dd,  $J=6.2$  Hz, 1H, H-3), 2.96-2.82 (m, 3H, H-4, H- $\alpha$ ), 2.29-2.45 (dd,  $J=4.8$  Hz, 1H, H-4), 1.25 (d,  $J=6.6$  Hz, H- $\beta'$ ); <sup>13</sup>C NMR ( $\text{CDCl}_3$ ):  $\delta$  154.17, 151.93, 145.84, 145.03, 132.89, 132.78, 131.07, 130.74, 128.33, 127.87, 126.83, 120.31, 114.87, 112.42, 60.92, 60.86, 59.43, 56.46, 39.21, 38.63, 23.48, 22.12.

## **Chapter 6**

### **Future Work**

#### **6.1 Further Examinations of the Isolation Process**

The isolation of bisbenzyltetrahydroisoquinolines and other alkaloids from the roots of *Mahonia aquifolium* (Oregon Grape) was not met with great success. Various extraction and purification procedures utilized were lengthy and did not lead to the isolation of the target compounds. Nonetheless, a great deal was learned that could serve to aid in future fractionation procedures. One such method involves the use of supercritical fluids as an alternative to the more traditional means of natural product extraction.<sup>71</sup> It is also recommended that LCMS (Liquid Chromatography Mass Spectrometry) be used to analyse the fractions obtained to aid in the identification of target constituents. HPLC (High Performance Liquid Chromatography) and the use of preparative chromatography columns with suitable solvent systems could also be employed to isolate target constituents.

#### **6.2 Further Investigations into the LOX Inhibition and Keratinocyte Anti-proliferation Assays**

As described in Section 3.3.2, determination of agonistic relationships between constituents of *M. aquifolium* was attempted by performing LOX inhibition assays on alkaloidal fractions obtained from silica gel column chromatography. This analysis did not meet with much success considering that the alkaloids contained in each fraction could not be defined. By analysing these fractions on the LCMS, those with molecular

masses that are in the range of the target BBIQs can be further fractionated and tested for anti-inflammatory and antioxidation activities. This would allow for a more efficient means by which to separate effective and non-effective compounds.

Interest also lies in the mechanism of action by which extracts and individual alkaloids may act in biological systems. Previous examinations have focussed on the use of radioactive isotopes, stable isotope incorporation and/or enzymatic investigations, requiring the isolation and identification of metabolic intermediates and end products. Recent investigations have focussed on the NMR study of metabolic pathways in cell cultures without the incorporation of tracer labelling.<sup>72</sup> It is the assumption of this author that this may represent a unique methodology for examining the metabolism of the test compounds in the keratinocyte assay. This type of analysis could serve to answer many of the current questions surrounding structure-activity correlations of BBIQs, a feat that has not accurately been accomplished by any group to date.

### **6.3 Further Examinations of the DPPH Radical Scavenging Assay**

The investigations into the DPPH radical scavenging by BBIQs by far consumed the greatest time and effort by this author. Discrepancies in reports by other authors provided great confusion which has now been largely alleviated, lending to a better understanding and proposal of mechanistic rationale. With this being said, many recommendations for future analysis can be made.

As described in Chapter 3, much work was devoted to the determination of rate

constants for the oxyacanthine-DPPH reaction, which were affected by pH and the various solvents used. Reactions in other solvents and solvent systems that vary in a wide range of pH's should be examined. Furthermore, reactions in protic and non-protic neutral mediums could be conducted to allow better understanding of the possible effect of hydrogen bonding and other solvent effects on the DPPH radical and the BBIQ antioxidant.

In order to better understand the role of the tetrahydroisoquinoline structure in the radical scavenging, the following investigations could be conducted. The products of the reactions with DPPH should be isolated and characterized which could provide much information with respect to the reactive functional groups. In addition, other BBIQs with different substitution patterns, ether linkages, and stereochemical conformation should be examined. A specific example would be the use of cycleanine as discussed in Chapter 4. This symmetrical BBIQ has no other functional groups capable of reducing the DPPH molecule, therefore it would be a useful candidate for examining the role of the tetrahydroisoquinoline center.

Finally, each of these experiments would benefit from the use of a stop-flow apparatus, capable of instantaneous mixing of the reactants under thermostated conditions, to be measured by a spectrophotometer. Such a system would allow for determination of initial rates kinetics, thus providing more accurate results for the expression of  $n$  and  $k$ .



## **6.4 Further Studies into the Synthesis of Cycleanine**

It should be considered that any current or future advances towards the synthesis of cycleanine can serve to aid in the construction of other BBIQs. As it stands, isoquinoline ring closure and formation of diaryl-ether linkages still pose significant difficulties in the synthesis of these types of compounds. Thus, it is reasonable to consider that future endeavours towards the synthesis of cycleanine will be focussed around these two particular methodologies.

### **6.4.1 Future Proposals for Improvement in Formation of the Isoquinoline**

The Bischler-Napieralski cyclization has been demonstrated to be effective in the synthesis of the benzylisoquinoline monomer unit, albeit in low yield. This was attributed to the unprotected phenol and amine groups creating a zwitterion effect. Future attempts to raise the yield should evaluate the effect of substituting the phenol with more robust functional groups that can be cleaved selectively.

### **6.4.2 Further Examination of Diaryl-Ether Forming Reactions**

Thus far, the diaryl-ether linkages required to construct the macrocycle of cycleanine has resulted in failure. However, it should be pointed out that the methodology reported by Buchwald *et al.* contains many variants. By varying the choice of base, biphenyl ligand, solvent, temperature and palladium source could result in dramatically different outcomes of products, as demonstrated by Buchwald *et al.* themselves. A great deal of future work aimed at effecting diaryl-ether linkages, could be focussed on varying reaction conditions and examining their effect on product formation

or lack thereof. Furthermore, model studies on the coupling reaction of aryl halides with *ortho*-substituted electron-donating groups should be examined. Success utilizing this methodology would provide a novel feat that has not previously been reported in the literature.

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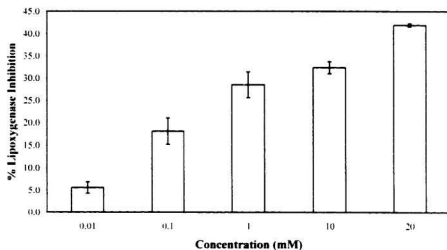
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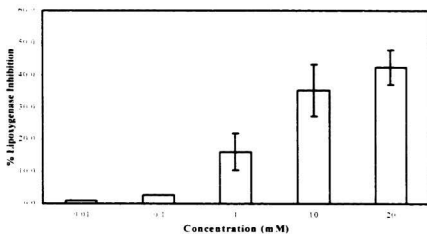
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## APPENDIX 1

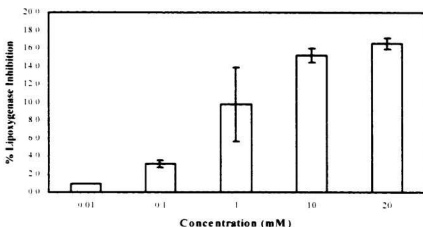


**Figure A-1.** Dose-responsiveness of oxyacanthine (**2**) on the inhibition of the action of lipoxigenase.

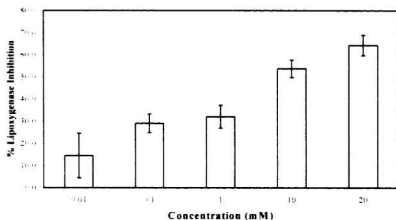


**Figure A-2.** Dose-responsiveness of berberine (**36**) on the inhibition of the action of lipoxigenase.

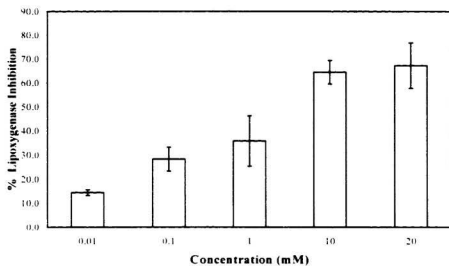




**Figure A-3.** Dose-responsiveness of oxyberberine (**39**) on the inhibition of the action of lipoygenase.



**Figure A-4.** Dose-responsiveness of the crude alkaloids on the inhibition of the action of lipoygenase.



**Figure A-5.** Dose-responsiveness of the EtOH extracts on the inhibition of the action of lipoxigenase.

## APPENDIX 2

$$n = \frac{C_0}{C} \left[ 1 - \frac{A_t}{A_0} \right] \quad (1)$$

The following derivation proves equation (1) to be an appropriate expression if the following assumptions can be obeyed: that the absorbance A at time "t" can be calculated according to equation (2).

$$A = \frac{A_0[\text{DPPH}]_t}{[\text{DPPH}]_0} \quad (2)$$

Solving for  $[\text{DPPH}]_t$  we can substitute into the expression for x and derive equation (3).  
x is the number of mols of DPPH reacted at time "t".

$$\begin{aligned} x &= \text{mol of DPPH reacted} \\ &= [\text{DPPH}]_0 - [\text{DPPH}]_t \\ &= \text{DPPH}_0 - \frac{A[\text{DPPH}]_0}{A_0} \end{aligned}$$

$$x = [\text{DPPH}]_0 \left[ 1 - \frac{A}{A_0} \right] \quad (3)$$

Since the initial concentrations of antioxidant (AH) and DPPH are known, and that the

abstraction of one hydrogen atom quenches one radical molecule.  $n$  equals the number of hydrogens. Therefore, the concentration of the antioxidant at time  $t$  "[AH]" would be equal to the initial concentration less  $x n$ .

$$[AH] = [AH]_0 - x n$$

Substituting equation (3) into the expression for [AH] followed by a few simple mathematical manipulations equation (4) is obtained.

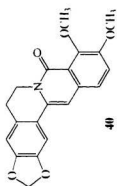
$$[AH] = [AH]_0 - \frac{[DPPH]_0}{n} \left[ 1 - \frac{A}{A_0} \right]$$

$$[AH]_0 - [AH] = \frac{[DPPH]_0}{n} \left[ 1 - \frac{A}{A_0} \right]$$

$$\boxed{n = \frac{[DPPH]_0}{[AH]_0 - [AH]} \left[ 1 - \frac{A}{A_0} \right]} \quad (4)$$

If  $n$  is calculated at the time that the plot levels out, the reduction of DPPH is no longer occurring, thus indicating the complete conversion of the antioxidant to an inactive oxidation product. Therefore at the final absorbance  $A_f$ , the concentration of AH should equal "0" and be solved.<sup>24</sup>

# APPENDIX 3



40



ppm

0

1

2

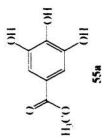
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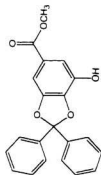
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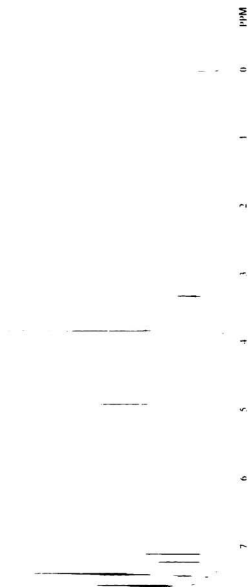
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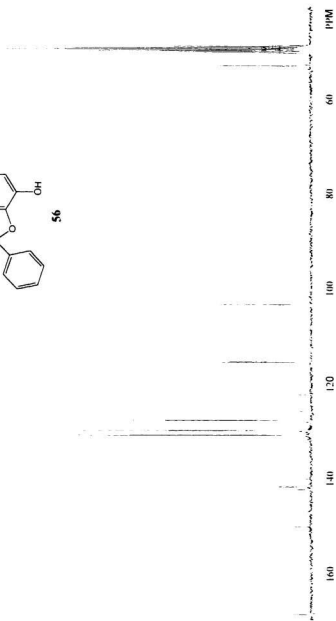
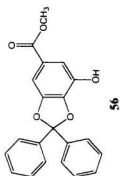
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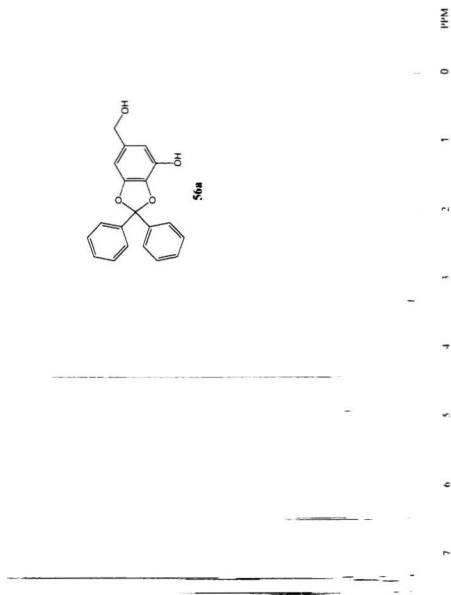


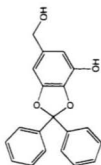
56











**56a**

ppm

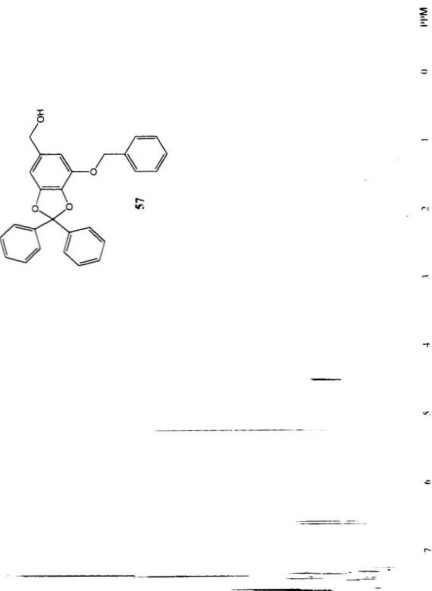
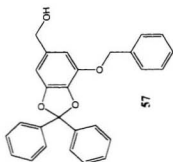
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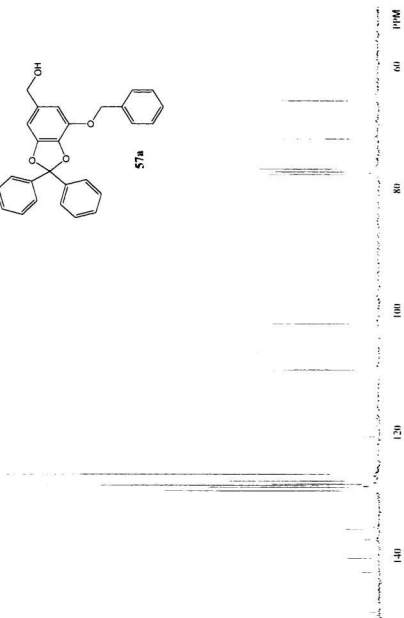
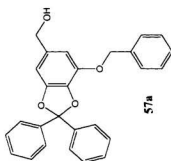
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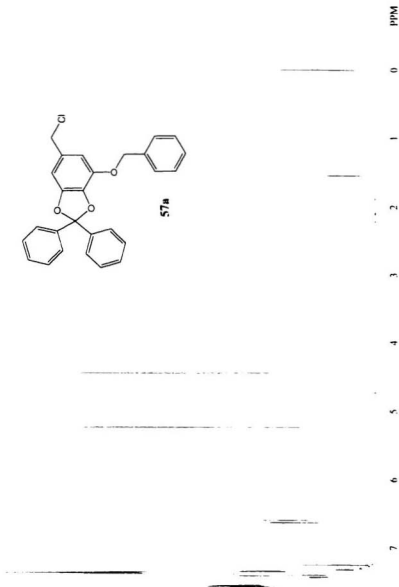
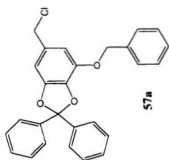
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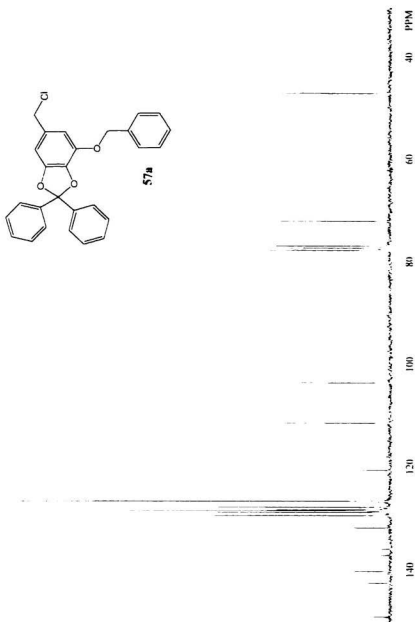
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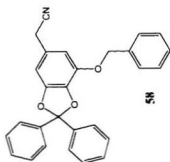
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**25**

PPM

0

1

2

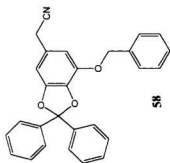
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5

6

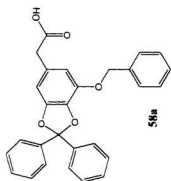
7



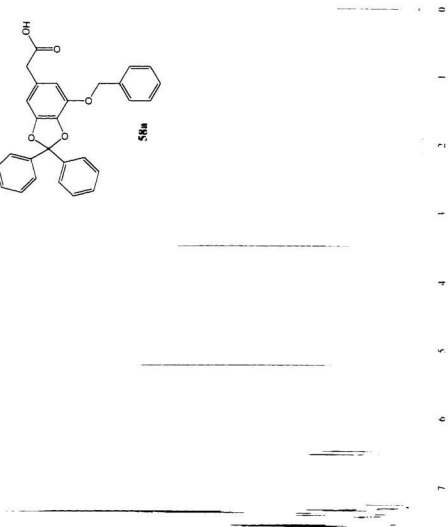
58

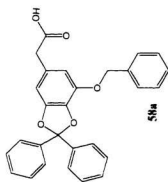


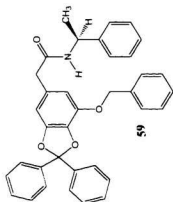




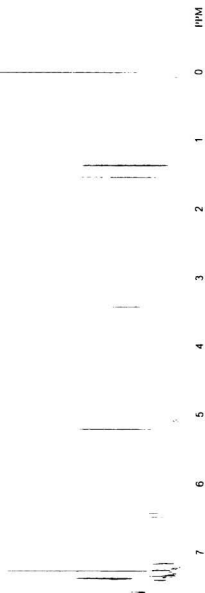
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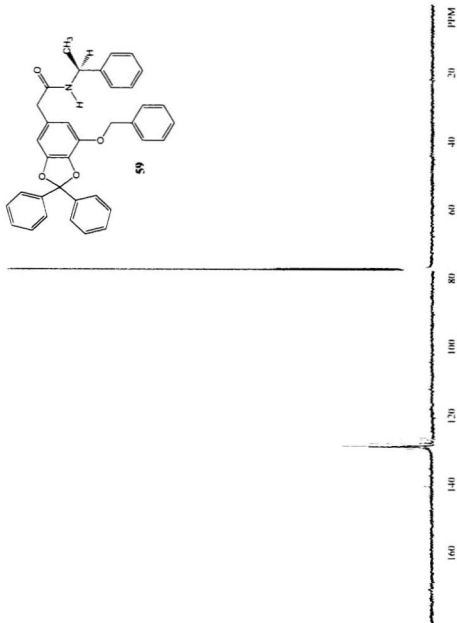


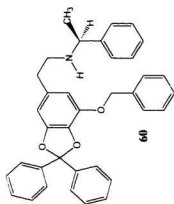




59







60

ppm

0

1

2

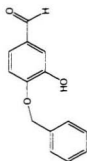
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4

5

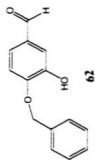
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7

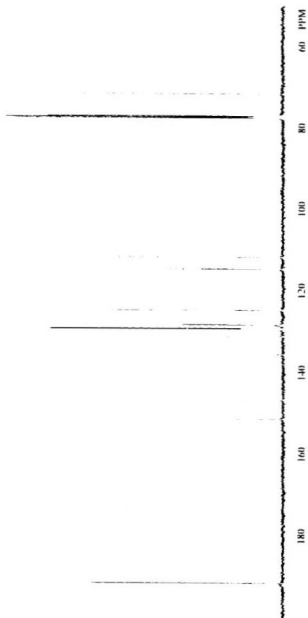


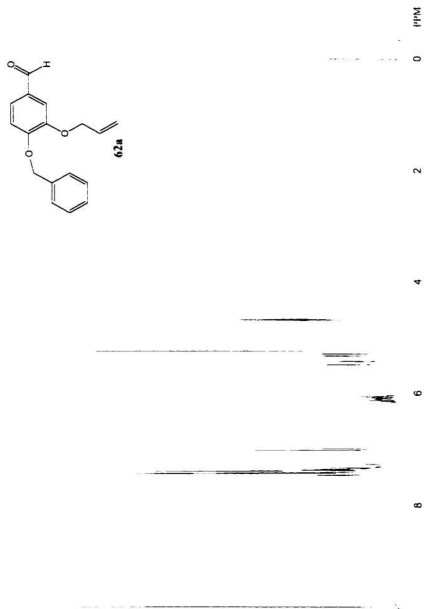
62



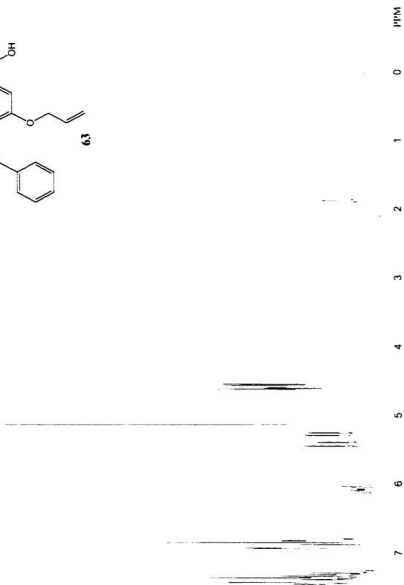
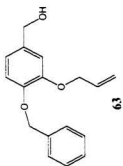


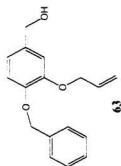
62

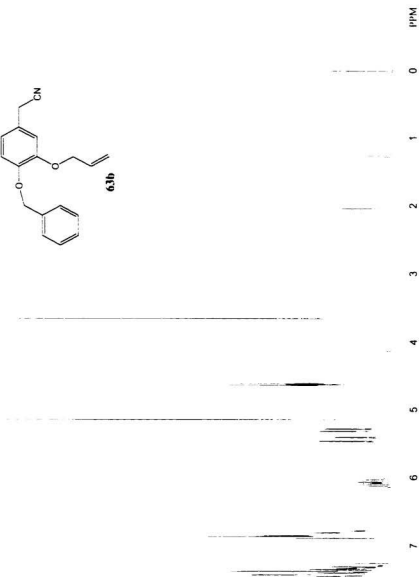
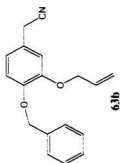


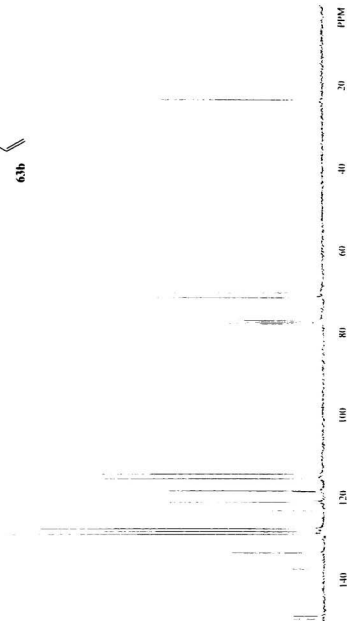
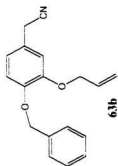


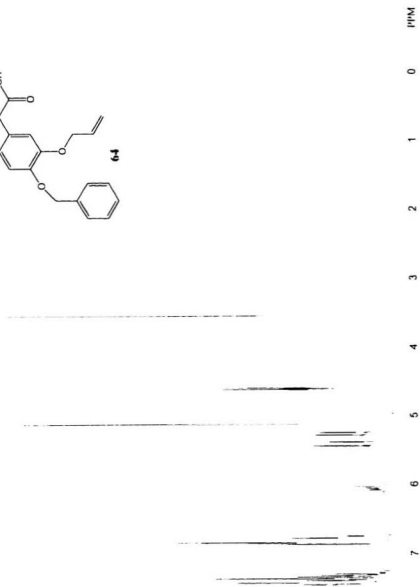
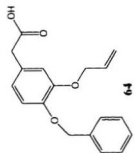


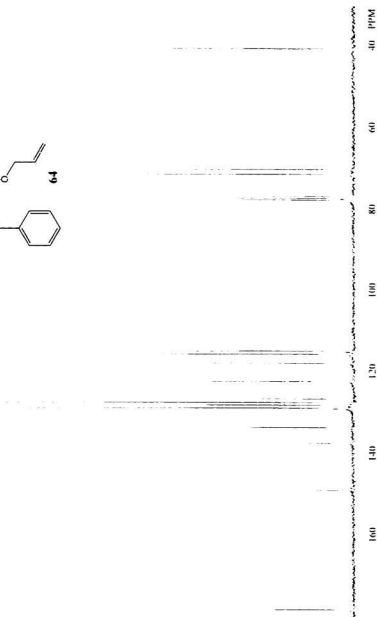
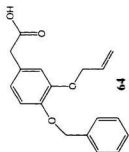


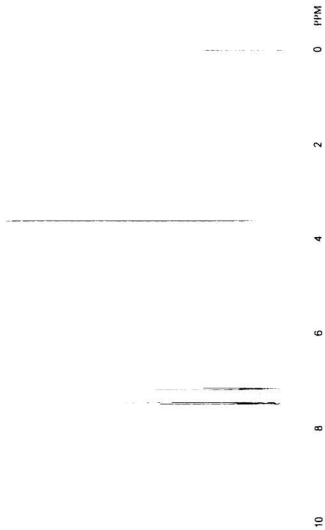
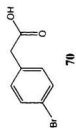


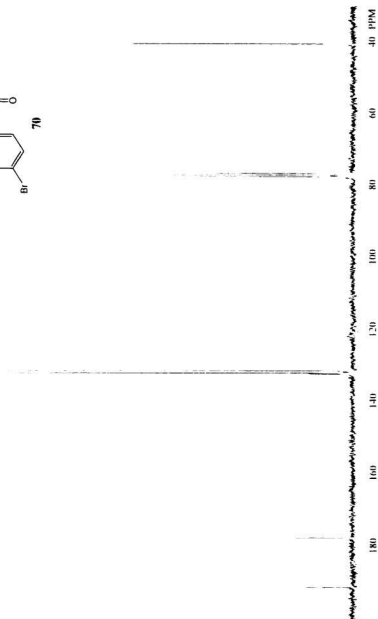
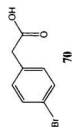




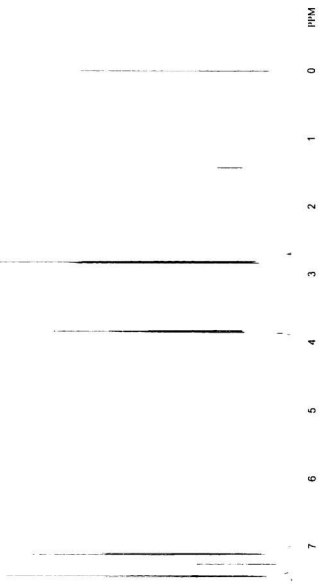
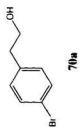


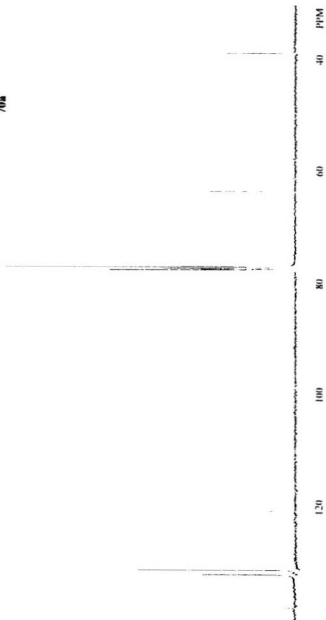
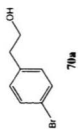


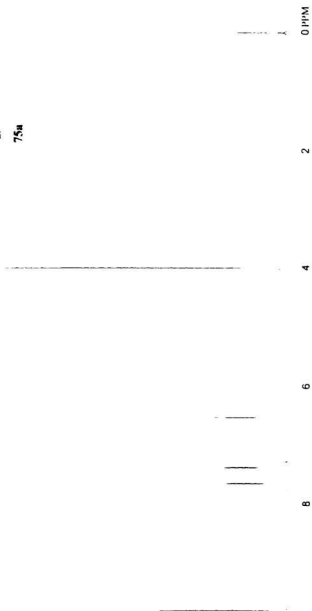
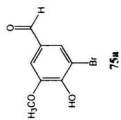


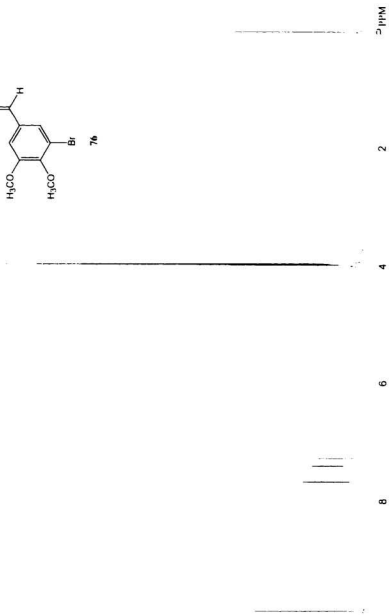
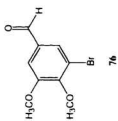


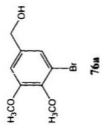


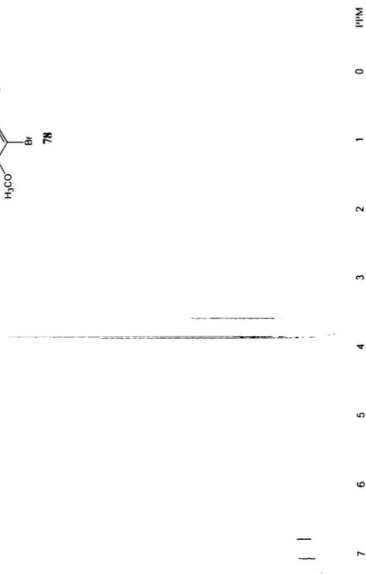
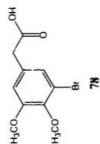


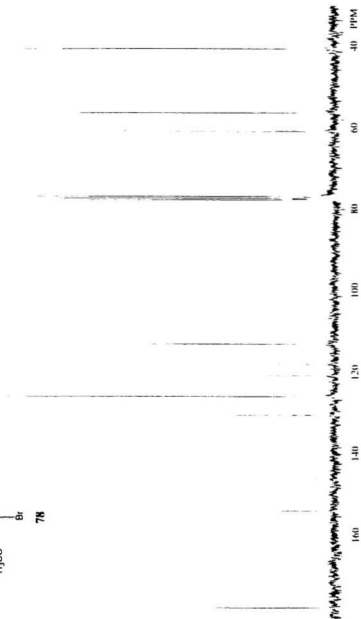
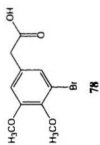


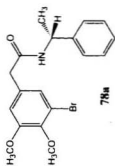




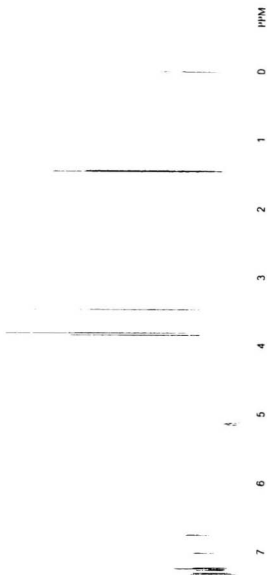




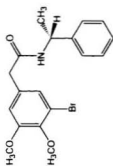




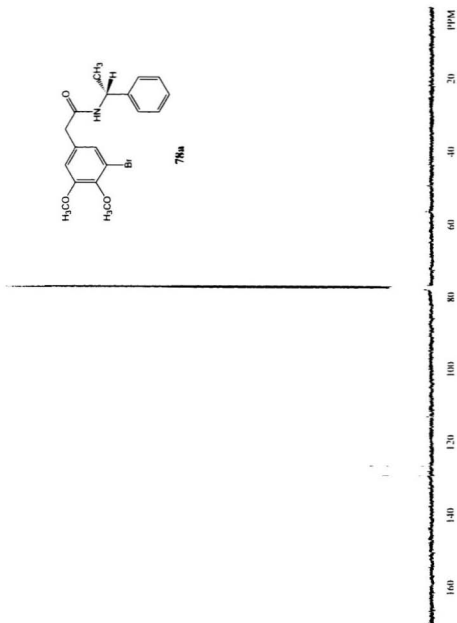
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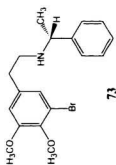




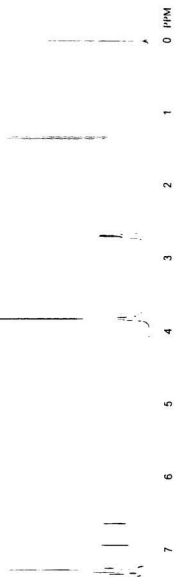


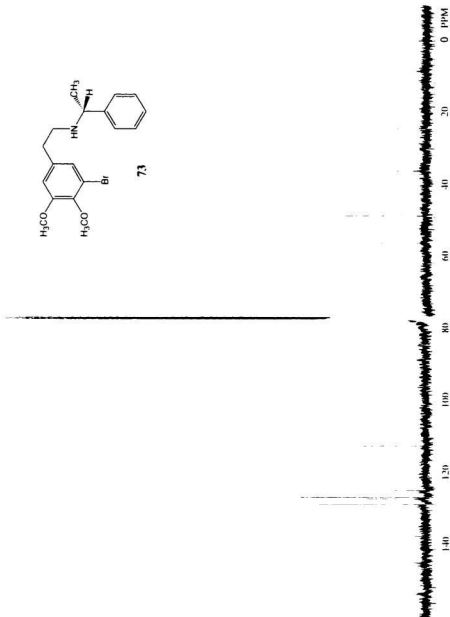
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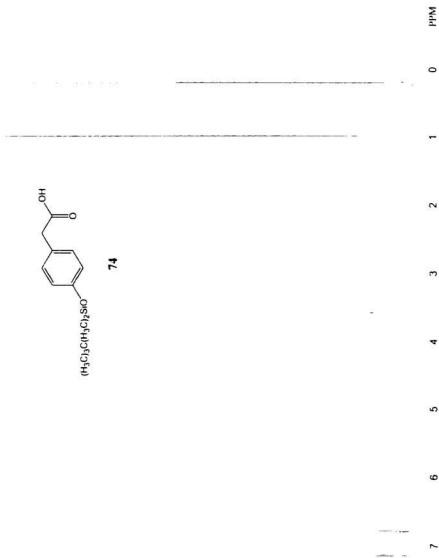


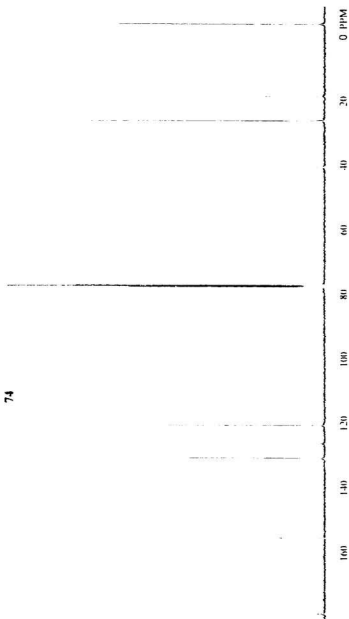
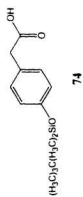


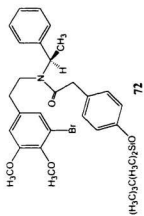
73











Chemical shift (ppm): 0, 1, 2, 3, 4, 5, 6, 7

Integration: 1.00, 1.00, 1.00, 1.00, 1.00, 1.00, 1.00

