ANTIOXIDANT PROPERTIES OF FLAXSEED LIGNANS USING *IN VITRO* MODEL SYSTEMS

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By

Farah Hosseinian

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University of Saskatchewan
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1.0 ABSTRACT

The major objectives of this study were to investigate the antioxidant properties of flaxseed lignans secoisolariciresinol (SECO **2)** and secoisolariciresinol diglycoside (SDG **1**) and their major oxidative compounds using 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH **47**) in an *in vitro* model of lipid peroxidation. This investigation was facilitated by the structural elucidation of the major oxidative compounds and the ability of flaxseed lignans to delay the onset of oxidation in two model systems.

This study showed that SECO **2** oxidation occurs at the aromatic (4-OH) and aliphatic (9-OH) hydroxyl groups. Conversely for SDG **1**, only compounds derived from the oxidation of aromatic hydroxyl groups were obtained because the 9-OH position is glucosylated.

SECO **2** oxidation with AAPH **47** showed that the intermediate **2a** is most likely involved in the generation of early-forming (**48** and **52)** and **2c** for the formation of late-forming **(49, 50** and **51)** oxidation compounds. Compound **48** is formed from dimerization of **2a** that is converted to **52** and then to **51**. Compound **50** was formed by the addition of a carbon-centre free radical of AAPH (AP radical) to **2c**. Compounds **50** and **51** trap carbon-centered AP radicals supporting SECO **2** as a chain-breaking antioxidant and AAPH **47** as a proper model for study of SECO **2** oxidation *in vitro*.

SDG **1** oxidation with AAPH **47** indicated that intermediates **1b** and **1c** are most likely involved for the formation of early forming compounds (**55** and **58)** and **1a** leads to the late forming compounds (**56** and **57)**. Compound **55** is a result of dimerization. Compound **56** may be directly formed via intermediate radical **1a** by adding AP free radicals. Compound **56** was a stable non-radical compound that could trap AP free radicals, thereby supporting SDG **1** as a chain-breaking antioxidant. Hydrogen abstraction from 4-hydroxyl yielded the radical **1a** and hydroxyl radical addition to **1a** yielded **57**. Compound **58** formed from the addition of $^{\bullet}OH$ or H_2O to **1c**.

This study demonstrated that AAPH **47** produces carbon-centred AP radicals upon thermal decomposition and mimics the formation of lipid peroxyl radicals. Interaction of carbon-centred AP radicals with SECO **2** and SDG **1** provides a good model to study the antioxidant reactions of SECO **2** *in vitro*.

The relative antioxidant capacity of the flaxseed lignans versus BHT **17**, in two model systems, was determined. The stoichiometric ratio for SECO **2** and SDG **1** were 1.5 and 1.1-1.2, respectively, compared to BHT **17** (2.0). The induction time by Rancimat analyzer measured inhibition of autoxidation mediated by flaxseed lignans SECO, SDG and SDG polymer in comparison with BHT **17**. The induction time data demonstrated that SECO **2** protected canola oil better than either SDG **1** or SDG polymer **3**.

These results are important for better understanding about the chemistry behind flaxseed lignan antioxidant activities. This study provided useful evidence that flaxseed lignans can be used as natural antioxidants.

This thesis is dedicated to………

….my parents
my brother, Vahid
my dear husband, Javad and
my loving children, Hamed and Saeed

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ABBREVIATIONS……………………………………………………Page

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride	iii
ABAP	2,2-azobis(2-methylpropionamidine) dihydrochloride	28
ABC	Three spin-coupled systems	111
ACN	Acetonitrile	88
ADP-ribosylation	Polyadenosine diphosphate ribose synthesis	44
ALA (18:3, ω-3)	α-Linolenic acid	1
AMVN	2,2'-azobis(2,4-dimethylvaleronitrile)	47
AOM	Active oxygen method	86
AP radical	Carbon-centre free radical of AAPH	iii
apo B.	Apolipoprotein B	20
BHA	Butylated hydroxy anisole	54
BHT	Butylated hydroxy toluene	31
CHD	Coronary heart diseases	19
CL	Chemiluminescence	30
COSY	Correlation Spectroscopy	91
COX-1, COX-2	Cyclooxygenases	37
CVD	Cardiovascular disease	18
CYP450	Cytochrome P-450	55
DEPT	Distortionless Enhancement by Polarization Transfer	91
DHA	Docosahexaenoic acid	2
DHBA	Dihydroxybenzoic acid	30
DLPC	1,2-Dilinoleoyl-sn-Glycero-3-phosphocholine	82
2D-NMR	2-Dimensional-NMR	91
DPPH	2,2-diphenyl-1-picrylhydrazyl	28
EC	(-)-Epicatechin	63
ECG	(-)-Epicatechin-3-gallate	64
EGC	(-)-Epigallocatechin	63

EGCG	(-)-Epigallocatechin-3-gallate	63
END	Enterodiol	8
ENL	Enterolactone	8
EPA	Eicosapentaenoic acid	2
ES-MS	Electrospray-mass-spectrometry	5
FRBR	Fenton reaction based radical assay	52
FRAP	Ferric reducing/antioxidant power	50
GC	Gas chromatography	52
GC-MS	Gas chromatography-mass spectrometry	28
GI	Gastrointestinal	8
GRAS	Generally Recognized as Safe	78
GPx	Glutathione peroxidase	56
GSH	Glutathione	55
GSSG	Oxidized glutathione	56
GSTs	Glutathione S-transferases	56
H_2O_2	Hydrogen peroxide	36
HDL-C.	High-density lipoprotein-cholesterol	18
Hep-G2 cells	Human hepatocarcinoma cells	25
HMBC	Heteronuclear Multiple Bond Correlation	91
HMGA	3-hydroxy-3-methylglutaric acid	3
HMG-CoA reductase	Hydroxy-methylglutaric CoA reductase	59
HMQC	Heteronuclear Multiple Quantum Correlation	91
HPLC	High performance liquid chromatography	82
IGF-I	Insulin-like growth factor I	22
IT	Induction time	86
$L^\bullet$	Lipid radical	45
LC-MS	Liquid chromatography-mass spectrometry	28
LDL-C	Low density lipoprotein-cholesterol	19
LOXs	Lipoxygenases	37
MAT	Matairesinol	3

MRFIT	Multiple Risk Factor Intervention Trial	18
MW	Molecular weight	4
NMR	Nuclear magnetic resonance	5
$NO^{\bullet}$	Nitric oxide	36
$NO_2^{\bullet}$	Nitric dioxide	36
NOESY	Nuclear Overhauser Effect Spectroscopy	91
NZ	New Zealand	23
$^1O_2$	Singlet oxygen	36
$O_2^{\bullet -}$	Superoxide anion	36
OCl^-	Hypochlorite	41
$OH^{\bullet}$	Hydroxyl radical	36
$OONO^-$	Peroxynitrite	36
ORAC	Oxygen radical absorbant capacity	50
PAF	Platelet-activating factor	20
PDA	Photodiode array	88
PG	Propyl gallate	54
PLR	Pinoresinol lariciresinol reductase	7
PMNL	Polymorphonuclear leukocytes	30
PUFA	Polyunsaturated fatty acids	37
PV	Peroxide value	51
$R^{\bullet}$	Alkyl radicals	41
$RO^{\bullet}$	Alkoxyl radical	36
$ROO^{\bullet}$	Peroxyl radical	36
ROOH	Alkyl peroxides	41
ROS	Reactive oxygen species	35
RP	Reverse phase	88
SDG	Secoisolariciresinol diglycoside	iii
SECO	Secoisolariciresinol	iii
SHBG	Sex hormone binding globulin	25

SOD	Superoxide dismutase	56
TAM	Tamoxifen	26
TBARS	Thiobarbituric acid reactive substances	52
TBHQ	Tertiary butylhydroquinone	54
TEAC	Trolox equivalent antioxidant capacity	50
TFA	Trifluoroacetic acid	88
UGTs	UDP-glucuronosyltransferases	70
VLDL	Very-low-density lipoproteins	20
ZDF	Zucker diabetic fatty	23
ω-3	Omega-3	1
ω-6	Omega-6	2

2.0. LITERATURE REVIEW

2.1. Flaxseed, general description

Flax (*Linum usitatisimum* L.) is grown as either an oil crop or as a fibre crop, with fibre (linen) derived from the stem of fibre varieties and oil from the seed of linseed varieties.[1,2] The seed of flax is flat and oval with a pointed tip, and varies in colour from dark brown to yellow.[3] Depending on the cultivar and growing conditions, flaxseed contains 40-50% oil and meal, comprised of 23-34% protein, 4% ash, 5% viscous fibre (mucilage) and lignan precursors (9-30 mg per g of defatted meal).[4-8] Annual world production of flax was 3.06 million tonnes in 1999-2000 and Canada is the world's largest producer of flax (about 38% of total production).[9] Flax is currently the second most important oilseed crop in Western Canada and is grown primarily in the prairie provinces of Saskatchewan (70%), Manitoba (26%), and Alberta (4%).[9]

2.2. Flaxseed oil

Flaxseed averages about 41% fat on a dry basis. It is low in saturated fat (9% of total fatty acids), moderate in monounsaturated fat (18%) and rich in polyunsaturated fat (73%).[10] α-Linolenic acid (ALA, 18:3, ω-3), an essential fatty acid, constitutes approximately 59% of the total fatty acids in flaxseed, making it the leading source of plant based ω-3 fatty acids.[5,11,12] The other polyunsaturated fatty acid in flax is linoleic acid (18:2, ω-6), that constitutes about 16% of the total fatty acids.[5,11] Flax is the richest source of ALA in the North American diet.[2] ALA is converted to two major metabolites

in vivo, the long-chain ω-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) after elongation and desaturation. The major metabolite of linoleic acid is arachidonic acid (20:4, ω-6).

2.3. Flaxseed mucilage

Flaxseed contains both soluble and non-soluble fibre, which accounts for about 28% of the weight of full-fat flax seeds. The proportion of soluble to insoluble fibre in flaxseed varies between 20:80 and 40:60.[13,14] The major insoluble fibre fraction in flaxseed consists of cellulose and lignin, and the major soluble fibre fraction are the mucilage gums.[2,15] Mucilage gums are a type of polysaccharide that becomes viscous when mixed with water or other fluids. These fibre fractions in flax can be classified as either dietary fibre or functional fibre. The classification depends on whether they are found intact in flax or are extracted from flax, purified and added to foods and other products. Thus, whole flax seeds and ground flax are sources of dietary fibre, while mucilage gums extracted from flax seeds and added to laxatives and cough syrups are a functional fibre.[2]

2.4. Flaxseed lignans

Lignans are diphenolic compounds of higher plants formed by the coupling of two coniferyl alcohol residues that are present in the plant cell wall.[16-21] In 1956, Bakke and Klosterman[22] isolated SDG **1** (Figure 2.1) from a fat free extract of linseed meal with a 3% yield. SDG **1** was found to be very soluble in water and alcohol. The same group isolated SECO **2** [2,3-di-(methoxy-4-hydroxybenzyl) butane-1,4-diol] (Figure 2.1)

by acid hydrolysis of SDG **1**.[22] SECO **2** is the aglycone (non-sugar) portion of SDG **1**. Both SDG **1** and SECO **2** have a UV absorption maximum at 280 nm, which is characteristic for lignans. Flax also contains small amounts of the lignans matairesinol (MAT) (11μg/g of full fat flaxseed), pinoresinol, pinoresinol diglucoside, isolariciresinol and a diastereomer of SDG **1**.[23-26]

SECO **2** is the major lignan present in flaxseed, which is found as the conjugate diglycoside SDG **1**.[27] Early studies demonstrated that SDG **1** was part of a larger complex.[22] An oligomer **3** (Figure 2.2) of SDG **1**, which is ester-linked via 3-hydroxy-3-methylglutaric acid (HMGA) **4**, has been identified.[16,24,28] A straight-chain oligomeric structure composed of five SDG **1** residues interconnected by four HMGA **4** residues (molecular weight ca. 4000 Da) was also reported.[29] SDG **1** may comprise greater than 35% (by weight or mole%) of the polymer.[29,30] Among foods, flaxseed is the richest source of SDG **1** (7 mg/g or 3.7 mg SECO **2**/g), which contains 75-800 times more SDG **1** than any other foods (Table 2.1).[23,31] Variation in the flaxseed lignan concentrations depends on the variety, location and year.[31]

Whole seed and ground flax typically contain between 0.7 and 1.9% SDG **1**, which is approximately 77–209 mg SDG **1**/tbsp of whole seed or 56–152 mg SDG **1**/tbsp of ground flax.[13] Flax oil containing added lignans has been available for several years. One such product contains 0.1% SDG **1** or about 14 mg SDG **1**/tbsp flax oil.[13]

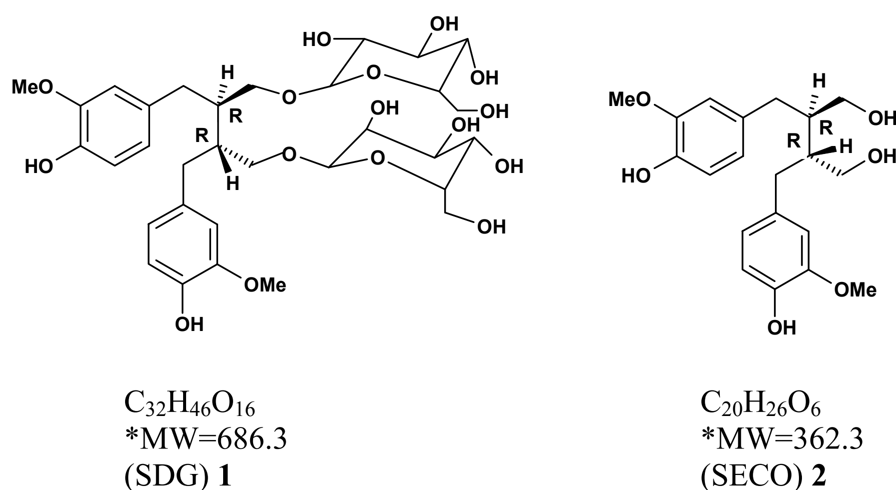


Figure 2.1. Structure of SDG **1** and SECO **2** (adapted from Muir and Westcott, 2003).[32]
*Molecular weight

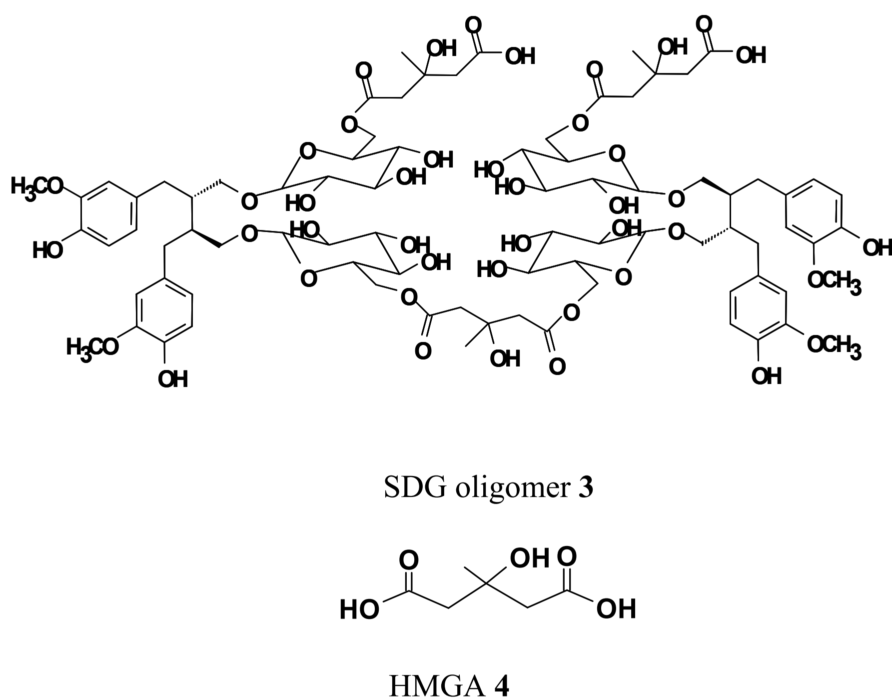


Figure 2.2. Structure of SDG oligomer **3** containing secoisolariciresinol diglycoside **1** and 3 hydroxy-3-methyl-glutaric acid units (HMGA) **4** (adapted from Westcott and Muir, 2003).[16]

Table 2.1. Lignan content of various foods (adapted from Morris, 2003).[13]

Food group/food	Seco 2 µg/g
Seeds	
Ground flax	3700.0
Pumpkin	213.7
Sunflower seeds	6.1
Cereals and grains	
Rye meal, whole grain	0.5
Oatmeal	0.1
Legumes	
Peanut	3.3
Soybean	2.7
Vegetables	
Broccoli	4.1
Carrots	1.9
Garlic	3.8
Berries	
Blackberry	37.1
Cranberry	15.1
Strawberry	12.1
Red currant	1.6

2.4.1. Properties of SECO 2 and SDG 1

Some basic physical properties of SDG **1** and SECO **2** in flaxseed are summarized in Table 2.2. SDG **1** and SECO **2** have been previously characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and electrospray-mass-spectrometry (ES-MS).[22,33]

Table 2.2. Some properties of SDG **1** and SECO **2** in flaxseed.[22,27,33,34]

Compound	Formula	*Molecular ion (m/z) $[M+H]^+$	Melting point (°C)	**Optical activity $[\alpha]_D$
(R,R)-SDG **1**	$C_{32}H_{46}O_{16}$	687.7	118.0	-2.0°
(R,R)-SECO **2**	$C_{20}H_{26}O_6$	363.3	114.0	+37.0°

*MW was measured using ES-MS (+ve ion mode).
Ethanol was used for SECO **2 and water was used for SDG **1**.

2.4.1.1. Optical rotation of SECO 2 and SDG 1

Stereoselectivity plays an important role in the late stage of phenylpropanoid metabolites (e.g., coniferyl alcohol) **5**. Naturally occurring lignans are often not racemic, but most are found in an optically active form (unlike lignins).[35,36] The enantiomer present can vary from one plant species to another. For example, in *Forsythia suspense* only (+) pinoresinol **6** was present, whereas in *Forsythia intermedia*, only (-) SECO **8** and (-) matairesinol **9** were reported (Figure 2.3).[35,37] In flax, SECO **2** is present essentially only as the (+) form **2**[22,27] and hydrolysis (10% HCl, 100°C) of purified SDG **1** gives enantiomerically pure (+) SECO **2** (Figure 2.4).[22,27] Studies on the biosynthetic routes to the formation of SECO **2** in flaxseed coupled with enantiomeric separation with chiral HPLC showed that the absolute configuration of (+) SDG **1** can mainly occur in the form of SDG **1** (R,R); however, some flax species showed (+) SDG **1** in the form of (S,S) configuration.[16,36] The absolute configuration of (-) SECO **2** has been determined via X-ray crystallography and NMR analysis using chiral shift reagents.[38]

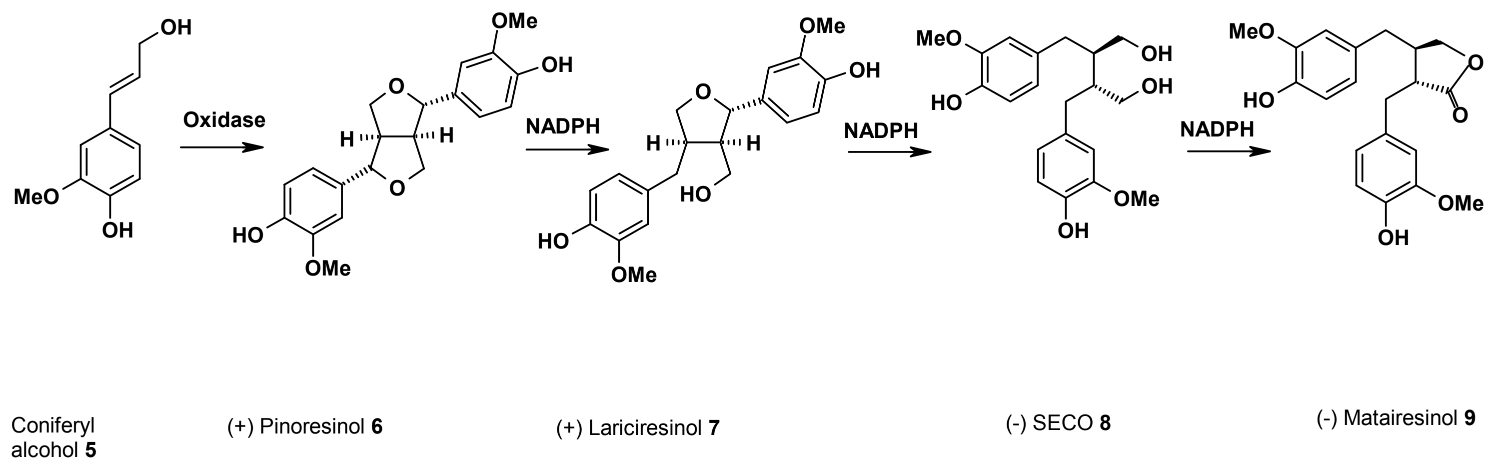


Figure 2.3. Biosynthetic pathway of (-) SECO **8** from *Forsythia intermedia*.[27]

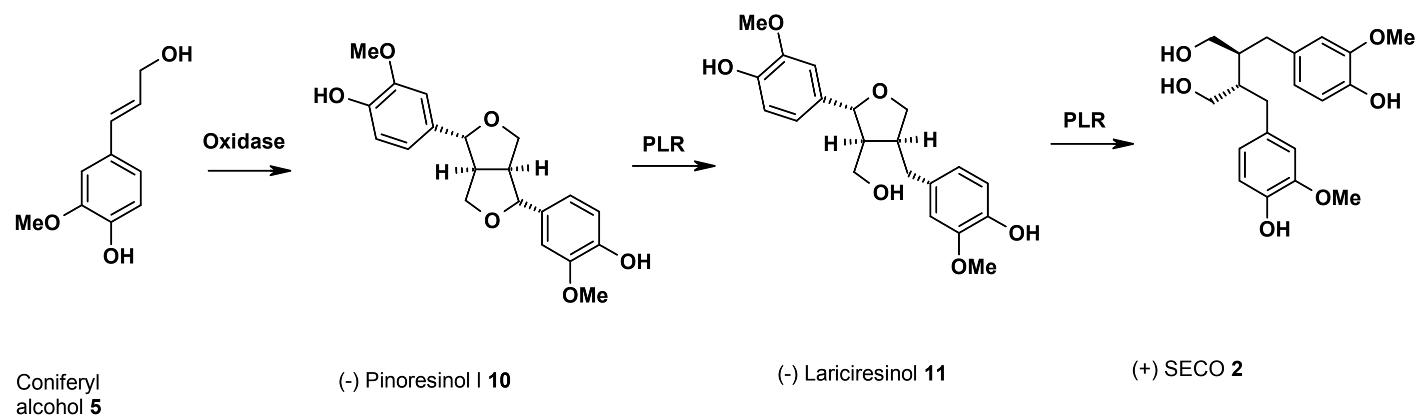


Figure 2.4. Biosynthetic pathway of (+) SECO **2** from flaxseed.[27] PLR: pinoresinol lariciresinol reductase

One problem in the literature regarding optical activity of lignans has been the reporting of a wide range of $[\alpha]_D$ for the same lignan in different plants. For example, the optical activity of SECO **2** has been reported as +37.0° (flaxseed), -7.0° (*Salvia plebeia* seed), and -30.8° (*Fitzroya cupressoides*).[22,39,40] Values of $[\alpha]_D$ = -35°, melting point 123°C has been reported for (-) SECO **8** (R,R)[38] and $[\alpha]_D$ = +37°, melting point of 114°C has been reported for (+) SECO **2**.[36]

2.5. Mammalian lignans

2.5.1. Conversion of plant lignans to mammalian lignans

Studies in which flaxseed was fed to rats, monkeys, or humans have found that the urinary excretion of the lignans enterodiol (END) **12** and enterolactone (ENL) **13** are significantly increased in urine.[41-48] Excretion of END **12** and ENL **13** increased 3-to 285-times after flaxseed consumption (5-10 g daily for six weeks) in the urine of 18 healthy young women, 31 healthy postmenopausal women and six healthy young men.[49-51]

The first mammalian lignans, END **12** (MW = 302) and ENL **13** (MW = 298) were identified in humans and animals in 1980.[18] Mammalian lignans are formed in the human body in the gastrointestinal (GI) tract, where GI bacteria hydrolyze the sugar moiety of SDG **1** to release SECO **2**.[6,8,27,28,52] This is followed by dehydroxylation and demethylation by colonic microflora to give the mammalian lignan END **12** (Figure 2.5). END **12** is presumed to be oxidized by the GI microbial flora to give ENL **13**. The ENL **13** may also be formed directly from

matairesinol **14** although this is likely to be a minor metabolic route if other lignans are present in the diet.[8,18,48,53-55] The mammalian lignans differ from plant lignans in that mammalian lignans have hydroxyl groups at the 3' position while plant lignans have their oxygenated substituents at the 3' and 4' positions.[32,52,56] A scheme outlining the proposed biosynthetic pathway of END **12** & ENL **13** from the flaxseed lignans SECO **2** and SDG **1** is shown in Figure 2.5. Concentrations of mammalian lignans in urine are typically greater than in plasma, thus most analytical methods target the measurement of urinary lignan levels (sections 2.7 and 2.8 for details).[32]

2.5.2. Role of gut flora in the oxidation of plant lignans to mammalian lignans

Incubation of flaxseed by bacteria present in stools, at a concentration of 10^3-10^4 bacteria/g of stool resulted in the formation of END **12** and ENL **13**.[53] This study showed that plant lignans are converted into END **12** and ENL **13** and the conversion is not reversible, thus END **12** and ENL **13** cannot convert to plant lignans *in vivo*. Furthermore, incubation of flaxseed in a human faecal bacterial culture at 10^3-10^4 bacteria/g converted END **12** to ENL **13** but sterile faecal cultures could not.[18] These results suggest that human gut floras are responsible for the conversion of plant lignans to mammalian lignans. A time course study of the metabolism of SDG **1** by human faecal cultures shows initial demethylation (Figure 2.6), occurs prior (20-30h) to dehydroxylation (48h).[57] Urinary excretion of END **12** and ENL **13** has been used as an index of plant lignan intake.[58]

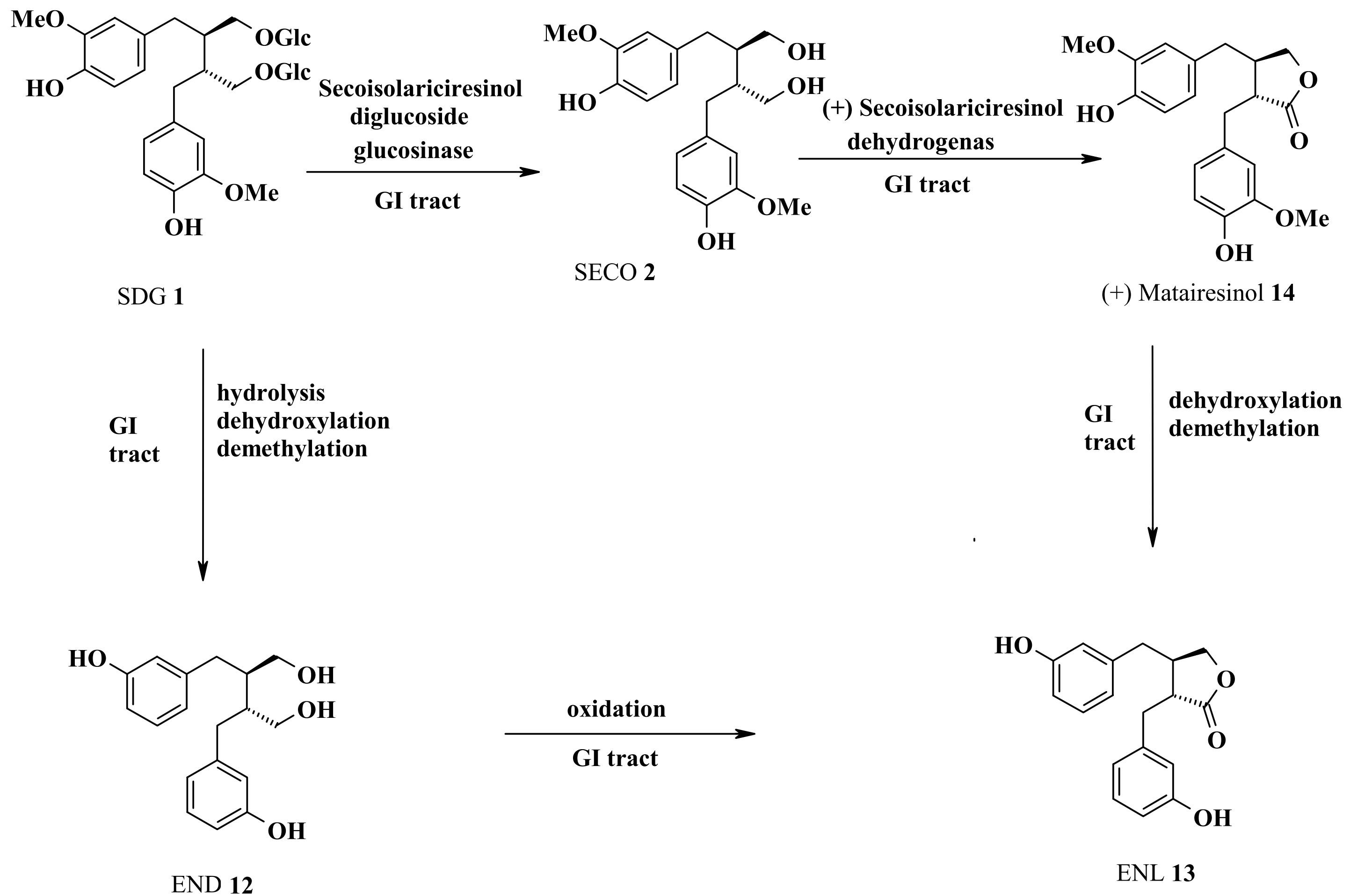


Figure 2.5. Biosynthesis pathway of flaxseed lignans SDG **1**, SECO **2**, and their corresponding mammalian lignans END **12** and ENL **13** (adapted from Ford *et al.,* 2001).[24]

SDG **1**

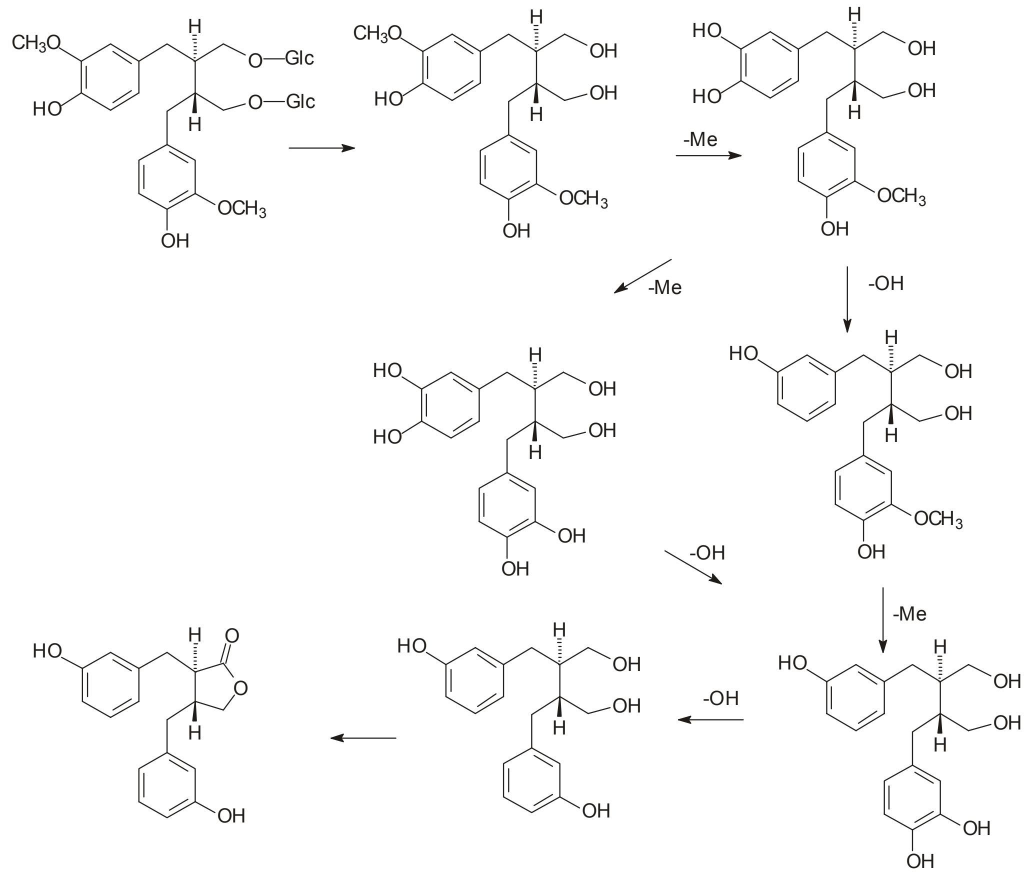


Figure 2.6. Metabolism of SDG **1** by human faecal bacteria.[32]
-OH=dehydroxylation, -Me=demethylation

2.6. Hepatic metabolism of mammalian lignans

2.6.1. Phase I metabolism

Studies of the hepatic metabolism of the mammalian lignans END **12** and ENL **13** in rat, pig, and human liver microsomes found a series of mono-hydroxylated derivatives of END **12** and ENL **13**.[59] These metabolites have also been found in the bile and urine of rats receiving oral doses of END **12**, ENL **13** or flaxseed.[32] A total of six aromatic mono-hydroxylated metabolites of ENL **13** and the 3 corresponding metabolites of END **12** were detected (Figure 2.7–2.8) with hydroxylation occurring either ortho or para to the aromatic hydroxyl groups.[59,60] A series of aliphatic mono-hydroxylated metabolites were also detected. Only the aromatic hydroxylated metabolites were found in human (female and male) urine, after 5 days of flaxseed (25 g/day) consumption.[61] The biological significance of these metabolites remains to be established.[61]

2.6.2. Phase 2 metabolism

Mammalian lignans are found in urine as the glucuronide and sulfate conjugates.[62] Once generated in the colon, END **12** and ENL **13** are absorbed and transported to the liver where they are conjugated mainly with glucuronic acid or sulfate.[21,27,58,62-64] The conjugated lignans are then excreted back into the digestive tract via the bile.

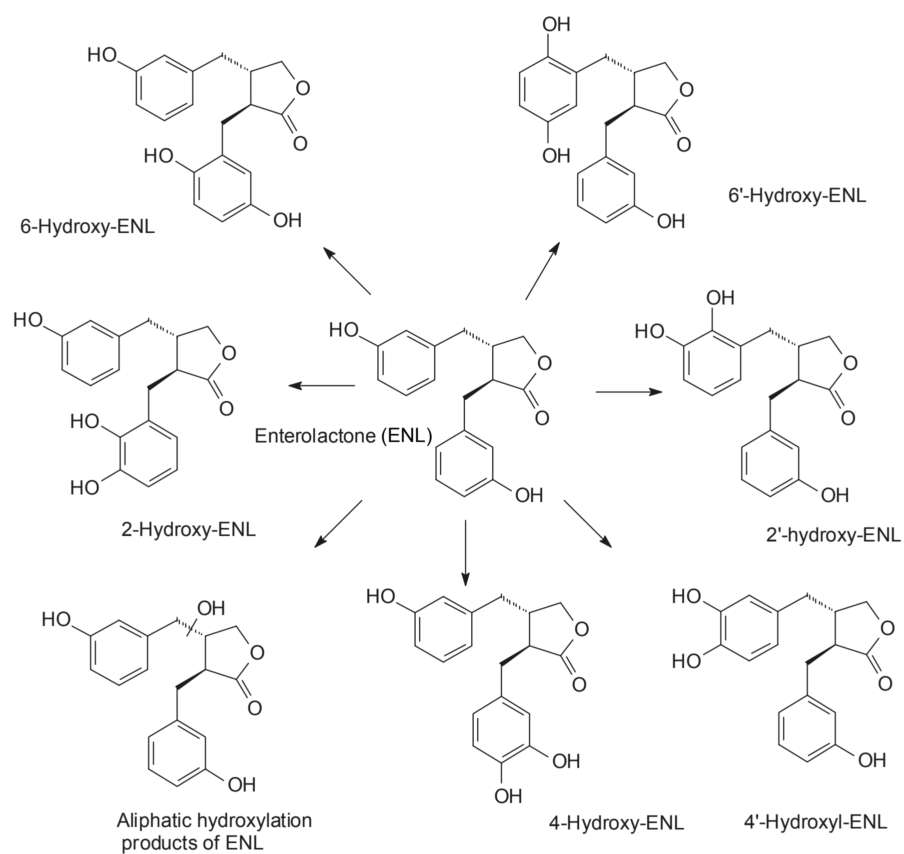


Figure 2.7. Oxidation metabolites of ENL **13**.[32,65]

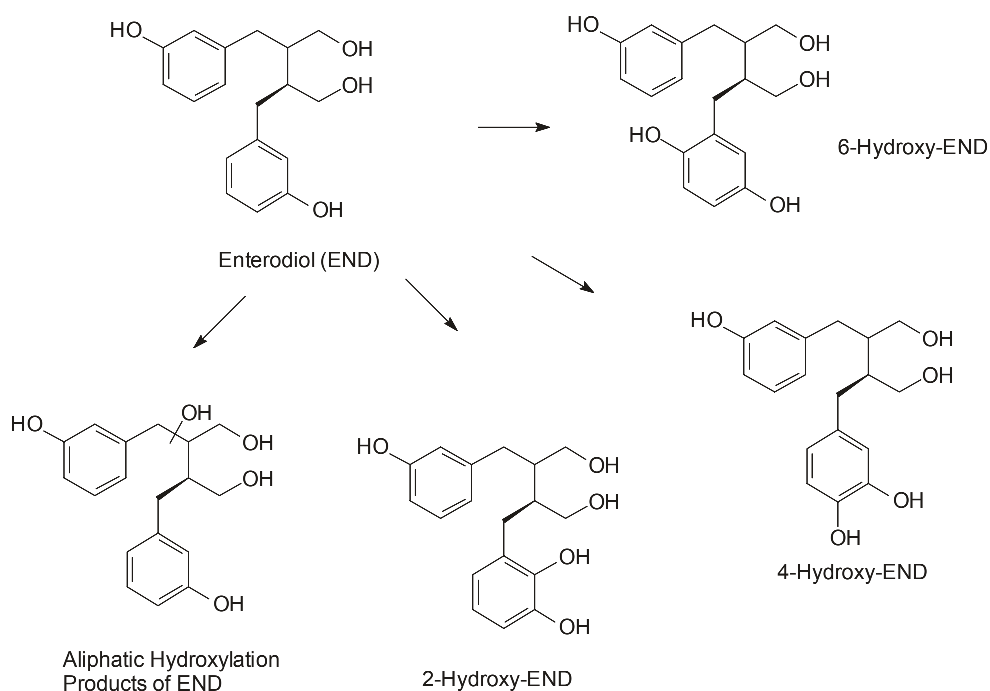


Figure 2.8. Oxidation metabolites of END **12**.[32]

A portion reaches the kidney and eventually is excreted in the urine as conjugates.[58] Conjugated lignans are reabsorbed from the intestine and the portion which escapes reabsorption becomes deconjugated during passage through the intestine by the action of bacterial β-glucuronidase and is excreted in the faeces in the unconjugated form.[24,62] Therefore, lignans extracted in bile and urine are mainly conjugated glucuronides[62,66] whereas the faeces contain the unconjugated forms of the lignans.[67] This enterohepatic circulation pathway of plant lignans is outlined schematically in Figure 2.9.

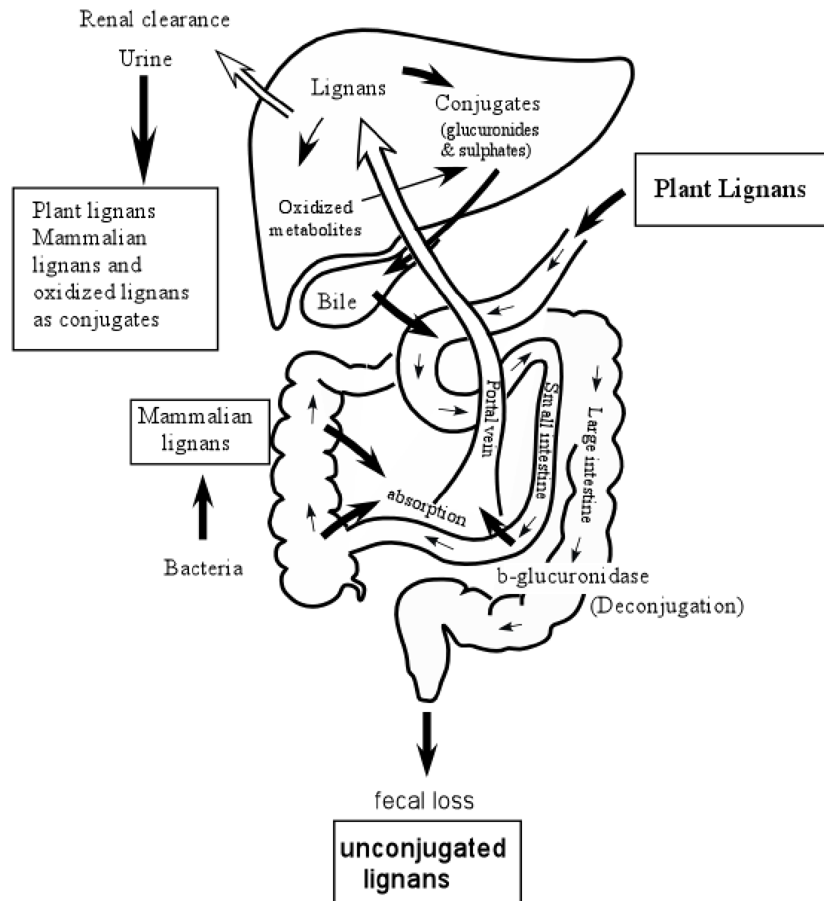


Figure 2.9. The enterohepatic circulation of plant lignans, their colonic and liver metabolites in man (adapted from Setchell *et al.,* 1982).[64]

2.7. Flaxseed lignan uptake and urinary levels

Urinary END **12** and ENL **13** has been found in relatively large amounts (10-1000 times more than endogenous estrogens) in humans.[68-70] Mammalian lignan production, based on urinary lignan excretion, is linear up to a diet of 5-10%

defatted flaxseed or 4.4 μmol SDG **1**/day intake in rats, and up to 25 g/day flaxseed intake in humans.[42] Consumption of 5 or 10 g of ground flaxseed by post-menopausal women significantly increased urinary excretion of END **12** by 1,009 and 2,867 nmol/day, respectively.[51] Urinary excretion of ENL **13** increased by 21,242 and 52,826 nmol/day, and total lignans increased (END **12** + ENL **13** + matairesinol (MAT) **14**) by 24,333 and 60,640 nmol/day, respectively.[51] Excretion of MAT **14** was not significantly altered by flaxseed consumption. In human urine, ENL **13**, END **12**, and MAT **14** were mainly excreted as mono-glucuronides (73-94%), with lesser amounts of monosulfates, sulfoglucuronides, diglucuronides and disulfates.[66,62]

Approximately one third of the mammalian lignans are excreted via the faecal route, while concentrations of the plant lignans in faeces and in urine are usually negligible.[68,71,72] In most studies, the urinary excretion of ENL **13** ranged from 1.1 to 4.2 μmol/24 h.[68-70,72-74] Excretion of END **12** was reported to be approximately 10 % of the excretion of ENL **13**.[69,70] It was observed that when rats were treated with radiolabelled SDG **1** (^{3}H-SDG **1**), most of the radioactivity was excreted by 48 hours (feces = 60%, urine = 28-30%).[58]

2.8. Flaxseed lignan uptake and plasma levels

The plasma levels of END **12** and ENL **13** in rats fed 1.5 mg/day SDG **1** was estimated to be 1 μmol/L.[75] The plasma level of lignans in humans fed 1.5 μmol/kg/body wt SDG **1** was also estimated to be 1 μmol/L.[76] One study on nine

healthy young women who supplemented their diets with 5, 15, or 25 g raw or 25 g processed flaxseed (in muffins or bread) for 8 days, showed that the concentrations of END **12** (12 nmol/L) were doubled compared with ENL **13** (6 nmol/L).[43] The conversion rate of END **12** to ENL **13** was very similar at the beginning and end of the study, suggesting that microflora were able to metabolize only a certain amount of plant lignans to ENL **13**.[43]

2.9. Flaxseed health benefits

Flaxseed consumption exhibits potential health benefits that include reducing the risk of cardiovascular disease, cancer and diabetes.[6,16,18,48,52,67,73,77-91] Whole flaxseed (2 tbs) contains a number of potentially beneficial components (Table 2.3). Most of the health benefits are associated with ALA, lignans and mucilage fibre, which will be discussed in this thesis.

Table 2.3. Nutritional profile of whole flaxseed (2 tbs ≅ 15 g of flaxseed).[11,92]

Alpha linolenic acid (Omega-3)	1,710 mg
Linoleic acid (Omega-6)	480 mg
Oleic acid (Omega-9)	540 mg
Lignin fibre	1,003 mg
Mucilage fibre	200.6 mg
Lignan	13.6 mg

2.9.1. ALA in flaxseed - health benefits

α-Linolenic acid in flaxseed can reduce the risk of cardiovascular disease (CVD), osteoporosis, rheumatoid arthritis and cancer.[92-96] In most studies flaxseed oil or whole flaxseed was used as the source of dietary ALA to show the effect of ALA on humans and animals.

2.9.1.1. ALA: Animal studies

A 10% flax oil supplement fed to mice reduced the growth of mammary tumor cells.[97-99] ALA may also have beneficial effects in hypertension. Rats on oil-rich diets supplemented with flaxseed oil at 40% of energy, showed lower blood pressure on average than control[100] and Golden Syrian hamsters fed 7.5-22% whole flaxseed in their diet for 120 days showed reduced plasma cholesterol and elevated high-density lipoprotein-cholesterol (HDL-C) levels.[101]

The effect of flaxseed lignans and alpha-linolenic acid, on growth and metastasis of human breast cancer in a mouse model has been studied. Mice injected with human breast cancer cells and supplemented with whole flaxseed (10%) showed a significant reduction in tumor growth rate and reduction in total incidence of metastasis (45%).[102]

2.9.1.2. ALA: Human studies

The Multiple Risk Factor Intervention Trial (MRFIT) showed that increased ALA intake over eight years correlated with up to a 50% lower risk of death from

coronary heart diseases (CHD).[103] Clinical studies have shown that flaxseed oil supplements may lower plasma cholesterol in hypercholesterolemic men.[10,11,104] One study showed that the consumption of a flaxseed oil (60 mL) supplement for six weeks increased EPA levels in plasma triglycerides.[105] A decrease in blood platelet aggregation was also observed, suggesting ALA in flaxseed may alter platelet aggregation.[105] Alterations in platelet composition and function were observed in healthy young men consuming a low fat diet supplemented with flaxseed oil (40 g) versus sunflowerseed oil.[106] The EPA level was more than doubled in the group taking flaxseed oil but was unchanged in the sunflowerseed group.[106] Thromboxane B_2 concentration decreased 30% in immune cells of 28 healthy men consuming 1.75 tbsp of flax oil daily for four weeks.[107] Dietary flaxseed oil (14 g/d) has also shown 90% inhibition of pro-inflammatory cytokine production in patients with rheumatoid arthritis.[108]

ALA in flaxseed oil (10 g/day) as supplements or in the diet of early postmenopausal women decreased levels of inflammatory bone resorbing cytokines and slowed the rate of bone loss, while increasing calcium absorption, bone calcium, and bone density, suggesting a possible protective effect against osteoporosis.[93]

Ground flaxseed (50g/day) consumed over 4 weeks increased the average daily ALA plasma levels by about 10 times in healthy adults.[109] Consumption of 50 g of ground flaxseed daily for four weeks lead to reductions in serum total cholesterol (6-9%) and low density lipoprotein-cholesterol (LDL-C) (9-18%) in studies of healthy young adults,[10,109] men and women with moderately high levels of

blood cholesterol[110] and postmenopausal women.[111,112] Triglyceride levels were not affected by diets containing ground flax and high-density lipoprotein-cholesterol (HDL-C) increased by 18% in only one study.[11]

Apolipoprotein B (apo B) decreased by 6% in hyperlipidemic women and men consuming partially defatted flax in their diets[113] and by 19% when eight men with normal blood lipid levels consumed flax oil.[114] Serum apo B concentrations decreased 7.5% in 25 postmenopausal women who ate 40 g of ground flax daily for three months.[112]

2.9.1.3. Mechanism of action

ALA is a precursor of EPA and DHA, alters the fatty acid composition of cell membranes, inhibits the release of pro-inflammatory eicosanoids,[107] affects membrane fluidity and elasticity[92,93] and decreases apo B levels, the major protein in LDL-C and very-low-density lipoproteins (VLDL).[92,93] Because ALA can interfere with the metabolism and biological actions of the ω-6 fatty acids, ALA may block the actions of platelet-activating factor (PAF), the formation of arachidonic acid and the formation of potent eicosanoids.[92,93] This may occur as a result of decreased prostaglandin levels or decreased cytokine production.[115]

2.9.2. Mucilage - health benefits

Mucilage or soluble fibre in flaxseed may also lower serum total cholesterol and LDL-cholesterol in the same way as other soluble fibres in the diet[113,116] and it

has been suggested that the lipid-lowering effects of flax are the result of the mucilage gums.[92,93,113] Diets containing 50 g carbohydrate from flaxseed or 25g flaxseed mucilage decreased blood glucose by 27%.[10] In a study of 29 men and women with high blood cholesterol who ate muffins (4/day) made with wheat bran or with partially defatted flax (less than 10% fat) for three weeks, total cholesterol decreased by 5% and LDL cholesterol decreased by 8% on the partially defatted flax diet.[13] These study findings suggested the role for flax mucilage gums in lowering blood lipids is due to more than ALA.[113]

2.9.3. Flaxseed lignans – health benefits

While lignans are found in many plants, flaxseed is the richest source of SDG **1** (0.7 and 1.9% in ground flax).[13,86] SECO **2** and SDG **1** may possess chemopreventive properties in animals and humans[6,16,18,48,52,67,73,77-91] including the potential to prevent hormone sensitive cancers (e.g. breast, prostate and colon cancer), hypercholesterolemic atherosclerosis and diabetes.[21,27,63,78,87,88,91,117-119] A study of urinary lignan ENL **13** and END **12** excretion, an indicator of dietary lignan intake, was found to be 50% lower in women with breast cancer suggesting that flaxseed could have chemopreventive properties.[69] This observation lead to a series of *in vitro*, animal, and human experiments to obtain a better understanding of the role of flaxseed and its lignans on different types of cancer.[21,27,63,78,87,88,91,117] Because flaxseed lignans are the richest source of precursors for the mammalian

lignans END **12** and ENL **13**, most studies use whole defatted flaxseed or SDG **1** to assess the role of the mammalian lignans.

2.9.3.1. Animal studies

The beneficial effects of flaxseed supplementation in breast cancer,[77,82,120,121] colon cancer,[82,88] skin cancer[122] and lupus nephritis[123] have been demonstrated in rats and mice.[123] Feeding *Inbred* mice with 5% flaxseed flour supplement in a high-fat diet at the promotional stage of tumorigenesis, (7,12-dimethylbenz[a]anthracene-mediated) produced a 66% reduction in tumour size.[82,98] The number and size of mammary tumours significantly decreased compared with the control values in *Inbred* mice fed for two weeks with SDG **1** at 73, 147 or 293 μmol/kg.[99] Plasma insulin-like growth factor I (IGF-I) levels in rats, which are associated with increased breast cancer risk, are reduced by dietary supplementation of flaxseed or SDG **1** in a rat breast cancer model.[84]

Studies in Sprague-Dawley rats suggest that SDG **1** has an inhibitory effect on the early stages of tumor development in mammary tissue, whereas flax oil and ground flax primarily effect established mammary tumors at a late stage of tumor development.[77,82,124,125] A study of colon cancer in Sprague-Dawley rats treated with full fat or defatted flaxseed, observed a 50% reduction in tumour number and an increase in urinary lignan excretion and cecal β-glucuronidase activity.[88,125]

A mouse study on lung metastasis of melanoma cells compared the effects of defatted flaxseed diets (2.5, 5, 10%) vs. SDG **1** (73, 147, 293 μM SDG **1**/kg diet)

and showed similar reductions in lung tumors.[88] A diet supplemented with 5% flaxseed inhibits the growth and development of prostate cancer in a transgenic mouse model.[126] Male New Zealand (NZ) rabbits fed a diet containing SDG **1** (15 mg/kg body weight) for eight weeks, showed a slowed development of hypercholesterolemic atherosclerosis, 35% reduction in LDL, and 64% reduction in LDL/HDL.[127] Furthermore, dietary flaxseed (7.5 g/kg daily, orally) containing 2-3% α-linolenic acid reduced hypercholesterolemic arteriosclerosis in rabbits by 46%, suggesting the reduction in arteriosclerosis was not due to ALA but may be due to the lignan component of the flaxseed.[128] It was also reported that milled flaxseed low in ALA protects against the same cardiovascular risks as extracted flaxseed oil, but also reduces serum LDL cholesterol, suggesting that the lignans may be responsible for the additional health benefits of flaxseed.[113,127,129]

SDG **1** in drinking water (22 mg/kg body weight) has been shown to cause a 71% decrease in the development of type 1 diabetes in diabetic prone rats[119] and a 75% reduction in type 2 diabetes in Zucker diabetic fatty (ZDF) female rats (40 mg/kg body weight).[130]

2.9.3.2. Human studies

A study in postmenopausal women showed that the excretion of the mammalian lignans increased 3.0 to 285.0 times after flaxseed consumption (5.0-25.0 g/day).[50] Several epidemiological studies have indicated a correlation between a high level of urinary lignans and a low incidence of cancer, particularly hormone-

dependent cancers, such as breast and prostate cancer.[44,63,66,90,131] Case-control studies of pre- and post-menopausal women with newly diagnosed early breast cancer showed that there is a 64% reduction in breast-cancer risk among women with high levels of urinary ENL **13**.[51,89,118] Postmenopausal breast cancer patients or omnivores at high risk for breast cancer have 50% lower urinary excretion of END **12** and ENL **13**[68,120] than those at low risk (vegetarians).[81,120]

Most human studies use whole or milled flaxseed incorporated into baked products such as breads, muffins, cookies, or breakfast cereals. For example, women with newly diagnosed breast tumors who ate a daily muffin containing 25 g of ground flax experienced significant reductions in breast cancer cell proliferation and tumor growth compared with women who ate whole-wheat muffins. It has also been observed that postmenopausal women fed flaxseed experienced a reduction in hot flashes.[132]

Several lignans and the isoflavonoids were found to compete with estradiol **15** for binding to the rat uterine type II estrogen binding site (*i.e.*, bioflavonoid receptor).[133] Studies examining the effects of flaxseed consumption on urinary estrogen metabolite excretion in postmenopausal women found that ground flaxseed consumption (0, 5 or 10 g/day) increased urinary concentrations of 2-hydroxyestrone and the 2/16 α-hydroxyestrone ratio in postmenopausal women as compared with the control group.[50] Unlike 2-hydroxyestrone, the 16-α-hydroxyestrone metabolite of estradiol **15** induces genotoxic DNA damage and aberrant hyperproliferation similar to that induced by chemical carcinogens.[134] Flaxseed consumption (25 g

ground flaxseed) also increased urinary concentrations of 2-hydroxyestrone, and the 2/16 -hydroxyestrone ratio to a greater extent than soy consumption (25 g soy flour) in postmenopausal women.[135]

In a preliminary clinical study, 25 men with prostate cancer consumed ground flaxseed (30 g/d) for 4 weeks as part of a low-fat diet.[136] Prostate cancer cell proliferation decreased and apoptotic death of cancer cells increased in patients using ground flaxseed as compared to the control group. The mammalian lignans END **12** and ENL **13** may be partly responsible for this effect.[136,137]

2.9.3.3. *In vitro* studies

Cell culture studies using human MCF-7 estrogen-dependent breast cancer cells showed that low concentrations (0.1-10 μM) of ENL **13** induced DNA synthesis (as a marker of cell growth) by 150-235%.[138] However, ENL **13** at high concentrations (20-90 μM) resulted in a 50% inhibition of DNA synthesis.[138] It has been observed that END **12** and ENL **13** not only significantly stimulates sex hormone binding globulin (SHBG) production in Hep-G2 cells (human hepatocarcinoma cells) in culture, but also suppresses Hep-G2 proliferation.[138,134]

The inductive or inhibitory effects of the mammalian lignans may also be dependent on the presence of a stronger estrogen or antiestrogen.[85,139] In the presence of estradiol **15**, high concentrations of ENL **13** and END **12** are more antiestrogenic on estrogen receptor positive breast cancer cells, while at low concentration, they are more estrogenic.[85,138] Estradiol **15** has proliferative effects

on estrogen dependent cancer cells and, some antiestrogens may inhibit this effect.[85] Estradiol **15** (1 nmol/L) and ENL **13** (0.5-2 μmol/L) separately stimulated the proliferation of MCF-7 cells, but their combination always resulted in lower or no stimulation.[85] Concentrations of ENL **13** above 10 μmol/L significantly inhibited the growth of the cells suggesting a toxic effect. Chen *et al.,* (2003)[140] determined that END **12**, ENL **13**, and tamoxifen (TAM) **16**, alone or in combination, can inhibit metastasis in breast cancer cells.[140,141]

An *in vitro* study also showed that END **12** and ENL **13** significantly inhibited the growth of three human prostate cancer cell lines.[137]

2.9.3.4. Correlation between *in vitro* production and *in vivo* excretion of lignans

A study was performed to determine whether SDG **1** can account for all the lignan production from flaxseed and if a relationship between *in vitro* production and *in vivo* urinary excretion existed.[42] Rats were fed a high fat diet without/with 2.5, 5 or 10 g/100 g ground flaxseed or 1.1, 2.2 or 4.4 μM SDG **1**/day (equivalent to levels in the respective flaxseed diets) for 4 wk.[42] This study showed that lignan production was dose-related since urinary lignan excretion increased linearly with doses from 0-5 g/100 g flaxseed and 0.0-2.2 μM SDG. A similar trend was seen *in vitro* (using human faecal inoculums) for SDG **1**, resulting in a high correlation between *in vitro* production and *in vivo* excretion of lignans.[42] Theoretical urinary END **12** and ENL **13** from the SDG **1** present in flaxseed correlated with the actual

excretion in flaxseed-fed animals. However, urinary END **12** and ENL **13** of SDG **1**-fed rats was only 20% of levels of flaxseed-fed rats, indicating the presence of other precursors or incomplete conversion of SDG **1** to END **12** and ENL **13**.[42]

2.10. Lignan- mechanism of effect

In vitro studies and *in vivo* studies suggest that flaxseed lignans may have both non-hormonal (*i.e.* antioxidant) and hormonal (*i.e.* weak esterogenic/antiesterogenic) effects[51,52,58,68-70,73,78,85,88-91,131,142-147] making them strong candidates for a role as natural cancer protective compounds.[58,73,133,147,148] Epidemiological investigations support this hypothesis, because the highest levels of these compounds are found in food in countries or regions with low cancer incidence.[73,79,90,148]

2.10.1. Non-hormonal mechanisms

2.10.1.1. Antioxidant activity

Natural antioxidants can be classified as primary (chain-breaking) antioxidants, which can react directly with lipid radicals and convert them into stable products, or as secondary (preventive) antioxidants, which can lower the rate of oxidation by different mechanisms.[149] Primary antioxidants most often act by donating a hydrogen atom, while secondary antioxidants may act by binding metal ions able to catalyse oxidative processes, by scavenging oxygen, by absorbing UV radiation, by inhibiting enzymes or by decomposing hydroperoxides.[150] It is known

that different natural phenolic compounds function as both primary and secondary antioxidants by different mechanisms. Monitoring of either the decrease of the radical or the antioxidant, or the formation of products can be used for assessing the antioxidant activity.[149]

The antioxidant activities of SDG **1** was lower than END **12** and ENL **13** in both lipid and aqueous *in vitro* model systems.[151] The antioxidant activity was monitored by hydroxyl and peroxyl radical scavenging activity of SDG **1**, END **12** and ENL **13** using a lipid emulsion system and inhibition of deoxyribose.[151] In this study, the deoxyribose assay was used to evaluate the non site-specific and site-specific Fenton reactant-induced $^{\bullet}OH$ scavenging activity.[151] The degree of oxidation of Fe^{2+} to Fe^{3+} (in Fenton reaction) by peroxyl radicals measured by ammonium thiocyanate assay.[151]

The antioxidant activity of 15 different major lignans found in knotwood extracts including hydroxymatairesinol, secoisolariciresinol **2**, nortrachelogenin, pinoresinol, and lariciresinol was studied.[152] The radical scavenging capacity was measured by monitoring the scavenging of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABAP (2,2-azobis(2-methylpropionamidine) dihydrochloride. Reaction products were identified by GC-MS, LC-MS and NMR spectroscopy. Dimerisation of the two aromatic moieties was the major radical termination reaction.[152] This study found the reactions with DPPH resulted in more unidentified polymeric material than the corresponding reactions with ABAP. The formation of adducts was a predominant reaction in the experiments with ABAP.

Instead, polymerisation seems to be the dominating termination reaction with DPPH. Lignans with catechol (3,4-dihydroxyphenyl) moieties (e.g. dimethyl matairesinol) exhibited the highest radical scavenging capacity, while the corresponding guaiacyl (3-methoxy-4-hydroxyphenyl) lignans (e.g SECO **2**) showed a slightly weaker scavenging capacity.[152] In addition, the butanediol structure was found to enhance the activity, whereas a higher degree of oxidation at the benzylic positions decreased the activity. Additionally, the readily available lignans (−)-secoisolariciresinol **8**, a mixture of hydroxymatairesinol epimers and (−)-matairesinol **9** were studied in more detail, including kinetic measurements and identification of oxidation products in the reactions with DPPH and ABAP. Table 2.4, shows the radical scavenging capacity (EC_{50}) of lignans and reference compounds.

Table 2.4. Radical scavenging capacity (EC_{50}) of lignans and reference compounds, in order of radical scavenging capacity. DPPH/AH = stoichiometric factor at EC_{50}.[152]

Compound	DPPH/AH
Matairesinol	2.9
Hydroxymatairesinol	2.6
Secoisolariciresinol	4.5
7-OH-Secoisolariciresinol	3.1
Nortrachelogenin	2.3
Pinoresinol	2.3
Lariciresinol	2.3

Beneficial effects of SDG in cancer and lupus nephritis showed that these beneficial effects could be due to the ability of SDG to scavenge $^{\bullet}OH$ radicals.[153] $^{\bullet}OH$ was generated by photolysis of H_2O_2 (1.25 - 10.0 μmol/mL) with UV light and was trapped with salicylic acid which is hydroxylated to produce $^{\bullet}OH$-adduct products 2,3-dihydroxybenzoic acid (DHBA) and 2,5 DHBA. SDG showed that it has the ability to scavenge $^{\bullet}OH$ (using HPLC). SDG (25-2000 μg/mL) showed a concentration-dependent decrease in the formation of 2,3-DHBA and 2,5-DHBA. The decrease in $^{\bullet}OH$-adduct products was due to the scavenging of $^{\bullet}OH$ and not by scavenging of formed 2,3-DHBA and 2,5-DHBA. This study suggested that SDG scavenges $^{\bullet}OH$ and therefore has an antioxidant activity.[153]

The antioxidant activity of SECO **2**, SDG **1**, END **12** and ENL **13** have also been suggested to contribute in reduction of hypercholesterolemic, atherosclerosis and diabetes.[119,154] The antioxidant activity of SECO **2**, END **12**, and ENL **13** was investigated by Prasad,[154] using chemiluminescence (CL) of zymosan-activated polymorphonuclear leukocytes (PMNL). This study demonstrated that SDG **1**, SECO **2**, END **12**, ENL **13**, and vitamin E, at a concentration of 2.5 mg/mL, produced a reduction of zymosan-activated PMNL by 23.8%, 91.2%, 94.2%, 81.6% and 18.7%, respectively.[154] The antioxidant activity was highest with SECO **2** and END **12** and lowest with vitamin E. The relative antioxidant potency of SECO **2**, END **12**, ENL **13**, and SDG **1** was 4.9, 5.0, 4.3, and 1.3 respectively, as compared to vitamin E.[154] This study suggested SECO **2**, END **12** and ENL **13** are respectively 3.82, 3.95, and 3.43 more potent than SDG **1**.[154] Activation of PMNL is known to

generate oxygen free radicals. Oxygen free radical producing activity of PMNL was monitored by measuring luminol-dependent CL. The reduction of PMNL, therefore, reflects the antioxidant activity of the compounds studied.[154] Zymosan is a polysaccharide that is capable of stimulating inflammatory cytokine production and PMNL. Zymosan may serve as a model for the study of innate immune responses.[155]

Other plant derived lignan compounds such as sesamin and sesamolin (the major sesame seed lignans) and a series of related dibenzocyclooctene lignans from the fruit of *Schisandra chinensis* including schisandrin B have been demonstrated to exhibit antioxidant activity both *in vitro* and *in vivo*. For example, sesame lignans enhance antioxidant activity of vitamin E in lipid peroxidation systems.[156] Sesamol containing a free phenolic group inhibited lipid peroxidation in both the systems whereas sesamin and sesamolin having methylenedioxy groups were effective only in the microsomal system.[156] Since detoxifying enzymes are localized in microsomes, the inhibitory effects of sesamin and sesamolin observed in the microsomal system may be attributed to their metabolites. The inhibitory effects of lignans were lower than tocopherols and BHT.[156]

2.10.1.2. Hormonal mechanisms

Several mechanisms have been reported for hormonal actions of mammalian lignans which are described in this section.

The estrogens (in women) and testosterone (in men) are steroid hormones made from cholesterol by the sex organs. The mammalian lignans are believed to work by binding to estrogen receptors on cell membranes.[68,147,157] The bound mammalian lignans affect the receptors' actions within the cell. They can act as either estrogen agonists or antagonists. Estrogen antagonists oppose the actions of endogenous estrogens. When blood levels of the endogenous estrogens are high (as in premenopausal women) the lignans can bind to the estrogen receptor and block the actions of endogenous estrogens. In this case, they act as antagonists. After menopause, the levels of endogenous estrogens in the blood decrease naturally because the ovaries release less natural estrogens. In this case, the lignans act like weak estrogens.[52,58,135]

END **12** and ENL **13** may bind to estrogen receptors on SHBG, thus blocking the binding of estrogen and testosterone.[68,133,158] As SHBG is found in breast cancer cells, the binding of mammalian lignans to SHBG may interfere with cancer processes that are controlled by estrogen.[133] ENL **13** and END **12** may compete with hormones for binding to SHBG.[133,159,160] ENL **13** was reported to be 10 times weaker than estradiol **15** at stimulating SHBG and its *in vivo* concentration was found to be 100–10,000 times greater than estradiol **15**. Thus, ENL **13** might have a greater contribution to physiological regulation of SHBG than estradiol **15**.[120] Increased binding of lignans to SHBG may help the transport of lignans to target tissues.[78,159,161] Lignans may inhibit cell proliferation.[85,90,144,147,162-164] Human estrogen synthetase (aromatase) is responsible for the conversion of androgen to

estrogen.[146,52] ENL **13** is a moderate or weak inhibitor of aromatase and this lignan binds to or near the substrate binding region of the enzyme active site. This may lower the amount of available estrogen and increase the risk of breast cancer. The inhibition is competitive with respect to testosterone, the lignan affinity is 1/75-1/300 that of these natural substrates.[146]

ENL **13** is rapidly conjugated to its monosulfate.[61,62] It is suggested that one possible mechanism by which ENL **13** may affect the growth of estrogen sensitive cells is by competition of ENL **13** and its sulfate with the estrogens for sulfokinases and sulfatases involved in estrogen metabolism in the cells.[85] ENL **13** at low concentrations (1-10 μM) has a stimulatory effect on DNA synthesis and estrogen dependent breast cancer cells (MCF-7), whereas at high concentrations (30-33 μM) has inhibitory effects on these cells.[85] The stimulatory effects of mammalian lignans were found to be 10^3-10^6 times weaker than the endogenous estradiol **15**, the most potent estrogen produced in women.[83,139]

Prostate cancer, like breast cancer, is hormone-sensitive, and in the early stages of development, tumour growth is influenced by the sex hormone testosterone and its active metabolite, 16 α-dihydrotestosterone.[165] The chemical structures of flaxseed lignans are similar to estradiol **15** (regarding the distance between the two –OH groups) and tamoxifen **16** (Figure 2.10), a breast cancer drug, suggesting that they may have weak estrogenic/antiestrogenic properties.[52,144,145,162]

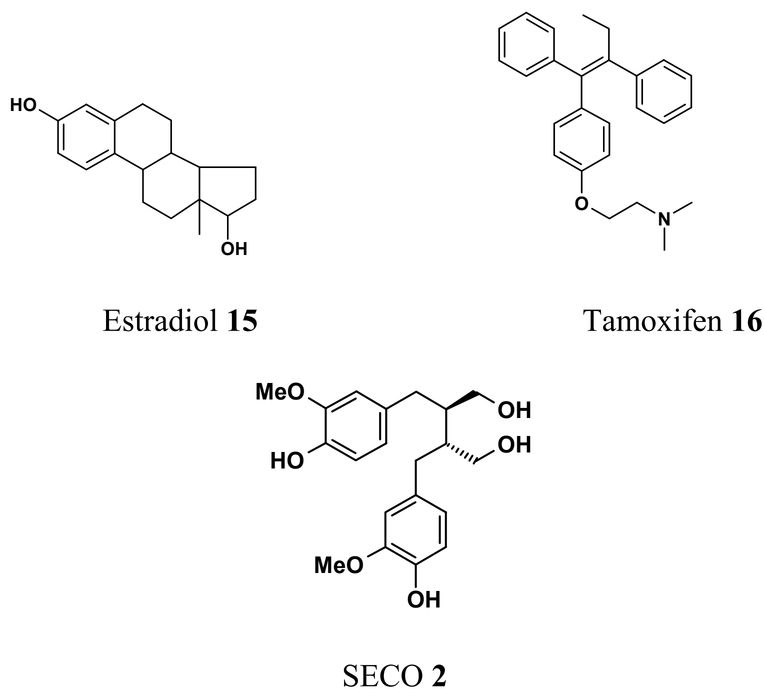


Figure 2.10. Chemical structure of estradiol **15**, tamoxifen **16** and SECO **2**, adapted from Lumsden, *et al.,* 1989.[145]

Decreased plasma IGF-I and epithelial growth factor receptor concentrations may be related, in part, to lower breast cancer risk.[84] IGF-I is a protein hormone similar in molecular structure to insulin. Flaxseed may inhibit human breast cancer growth and metastasis by downregulation of IGF-I and epithelial growth factor receptor expression.[102]

2.11. Free radicals-physiology

Free radicals are highly reactive substances formed in the body's cells as a result of metabolic processes.[166,167] In 1954 Gerschman *et al.,* first proposed the theory of free radical formation.[168-170] A free radical is a molecule with one or more

unpaired electrons in its outer orbital.[171] Many of these molecular species are oxygen (and sometimes nitrogen) centered. Oxygen free radicals and its non-radical products are associated with reactive oxygen species.[172,173]

Free radicals are highly reactive, unstable molecules that react rapidly with adjacent molecules via a variety of reactions including: hydrogen abstraction (capturing), electron donation and electron sharing.[171] For example, the process of capturing an electron involves reacting with a donor molecule, which loses an electron and is said to have been oxidized.[174] The oxidized donor molecule then has the capacity to oxidize other molecules and, thus, sets up a chain reaction (self-propagation reactions). Although free radicals play an essential role in the body, they also can react with DNA, protein or lipids in the cell membrane and cause damage.[175]

2.11.1. Reactive oxygen species-sources

Reactive oxygen species (ROS) are found intracellularly and extracellularly and may be produced endogenously or arise from exogenous sourses, i.e. taken in from the environment.[176,177] Important sources of endogenous free radicals include prooxidative enzyme systems (e.g. lipoxygenase), drugs and their metabolites, pollutants, and other chemicals and toxins.[178,179] While some of these are directly toxic, many others generate free radical fluxes via the very metabolic processes that the body uses to detoxify them. External sources such as sunlight and other forms of radiation can generate endogenous ROS, which can lead to a number of

diseases.[178,180] ROS can also be formed in food through lipid oxidation and photosensitizers exposed to light.[181]

2.11.2. Reactive oxygen species-classification

Reactive oxygen species are constantly being generated in the body, as a result of the normal metabolic processes.[182] Mitochondria, which consume more than 90% of the oxygen in aerobic living organisms, are the main source of ROS and free radicals. Approximately 1% to 5% of the oxygen consumed by mitochondria is reduced and converted to these reactive oxygen species.[183,184] ROS can be classified into oxygen-centered radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and oxygen-centered non-radical derivatives such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other common reactive species are nitrogen species such as nitric oxide ($NO^{\bullet}$), nitric dioxide ($NO_2^{\bullet}$), and peroxynitrite ($OONO^-$).[185-189]

ROS cause lipid oxidation, protein oxidation, DNA strand breaks, and modulation of gene expression.[185,189-192] ROS are involved in many diseases such as atherosclerosis, cancer, stroke, asthma, arthritis and other age related diseases.[172,193-196] Some of the biological damage caused by ROS in the human body is outlined in Figure 2.11.

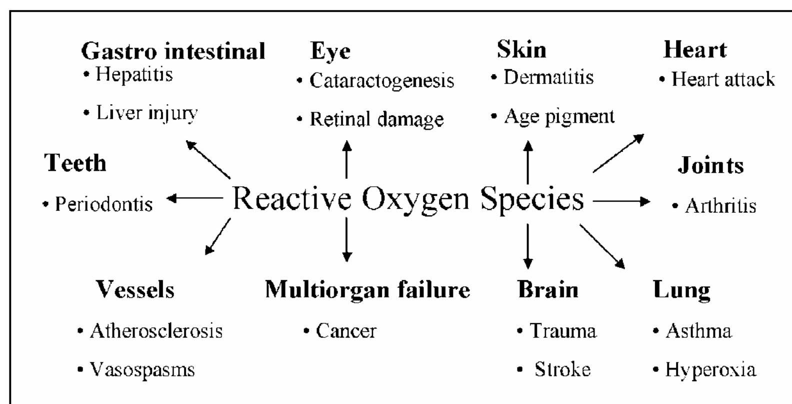


Figure 2.11. Biological damage caused by ROS in human body (Lee *et al.,* 2004).[197]

Because antioxidant defense in the human body is not completely efficient, increased free radical formation may produce a continuous level of oxidative damage.[170,172,178,184,186,187,190,191,193,196,198-201] Oxidative stress refers to a severe disturbance in the prooxidant-antioxidant balance in favor of the prooxidant, leading to potential damage.[177] The basic mechanisms of free radical formation are reviewed in the following sections.

2.11.3. Enzymatic formation of ROS

Lipoxygenases (LOXs), are found in plants and animals. LOXs are large monomeric protein with non-heme iron cofactor containing dioxygenases [cyclooxygenases (COX-1, COX-2)] catalyzing the oxidation of the polyunsaturated fatty acids (PUFA) forming hydroperoxides.[176,177,179,202] Substrates for LOXs in plants are linoleic acid (18:2) and linolenic acid (18:3) and in animals is arachidonic acid (20:4).[202] Because arachidonic acid either is not present in higher plants or is a

minor constituent of cellular lipids, plant LOXs are classified into 9- and 13-LOXs with respect to their positional specificity of linoleic acid.[177,179] In mammals, LOXs are classified into 5-, 8-, 12- and 15-LOX with respect to their positional specificity of arachidonic acid.[202] In mammals, lipoxygenase needs free forms of PUFA, which are not present in healthy tissue. PUFA (e.g. arachidonic acid) can be released from glycerides by membrane bound phospholipase.[203] Free PUFA are oxidized to form lipid hydroperoxides.[176,177,179] Lipoxygenase with Fe^{2+} ion exists in the inactivated status. Once Fe^{2+} is oxidized to Fe^{3+}, lipoxygenase can convert PUFA into hydroperoxides.[179] Lipoxygenase has been identified within atherosclerotic lesions, which suggests that this enzyme may be involved in the *in vivo* formation of lipid oxidation.[204]

2.12. Reactive oxygen species-mechanism

2.12.1. Superoxide anion ($O_2^{-\bullet}$)

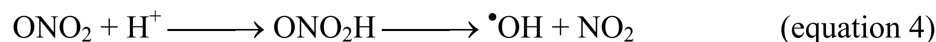
Superoxide anion and hydrogen peroxide are the main reactive oxygen species causing the oxidation of cells and tissues.[180,205] Superoxide anion itself is not a strong oxidant. Superoxide anion reacts with protons in water solution to form hydrogen peroxide (equations 1-3), which can serve as a substrate for the generation of hydroxyl radicals and singlet oxygen.[172,174,180,193]

$$2\,O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \qquad k\sim 0 \qquad \text{(equation 1)}$$

$$HO_2^{\bullet} + O_2^{-\bullet} + H^{+} \longrightarrow H_2O_2 + O_2 \qquad k\sim 10^{8}\ M^{-1}\ s^{-1} \qquad \text{(equation 2)}$$

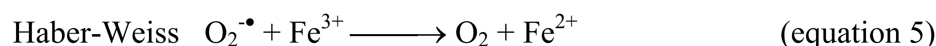
$$2HO_2^{\bullet} \longrightarrow H_2O_2 + O_2 \qquad k\sim 10^{6}\ M^{-1}\ s^{-1} \qquad \text{(equation 3)}$$

The superoxide anion can react with nitric oxide ($NO^{\bullet}$) and form peroxynitrite ($ONOO^{\bullet -}$). The conjugate acid of peroxynitrite, peroxynitrous acid, can further decompose to generate hydroxyl radical and nitric dioxide (equation 4).[185,204]

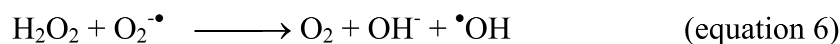


$$ONO_2 + H^+ \longrightarrow ONO_2H \longrightarrow {}^{\bullet}OH + NO_2 \quad \text{(equation 4)}$$

Superoxide can reduce Fe^{3+} to Fe^{2+}. The last two reactions are known as Haber-Weiss reaction (equations 5-6).



$$\text{Haber-Weiss} \quad O_2^{-\bullet} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad \text{(equation 5)}$$



$$H_2O_2 + O_2^{-\bullet} \longrightarrow O_2 + OH^- + {}^{\bullet}OH \quad \text{(equation 6)}$$

2.12.2. Hydroxyl radical (${}^{\bullet}OH$)

Hydroxyl radical is a highly reactive free radical (diffusion rate-limited) that can react with most living organisms.[197,206] Hydroxyl radicals react with lipids, polypeptides, proteins, and DNA.[175,206] Hydroxyl radical is highly electrophilic and can abstract electrons from proteins and polyunsaturated fatty acids[206-208] resulting in the formation of carbon centered free radicals ($R^{\bullet}$) (equation 7). The carbon-centered free radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical ($ROO^{\bullet}$) and alkoxy radical ($RO^{\bullet}$) (equation 8-10).[207]

$$RH + {}^{\bullet}OH \longrightarrow R^{\bullet} + H_2O \quad \text{(equation 7)}$$

$$RO^{\bullet} + O_2 \longrightarrow ROO^{\bullet} \quad \text{(equation 8)}$$

$$ROOH + Fe^{3+} \longrightarrow ROO^{\bullet} + Fe^{2+} + H^+ \quad \text{(equation 9)}$$

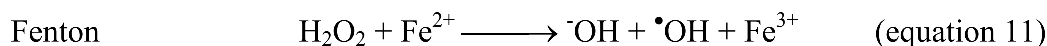
$$ROOH + Fe^{2+} \longrightarrow RO^{\bullet} + {}^{-}OH + Fe^{3+} \quad \text{(equation 10)}$$

Hydroxyl radicals can damage cell membranes and lipoproteins (the particles carrying cholesterol and triglycerides in the blood stream) by lipid peroxidation. Lipid peroxidation occurs by a radical chain reaction, i.e. once started, it spreads rapidly and affects a great number of lipid molecules.[170,198,200,209] For example, linoleic acids are mainly located in glycerolipids and phopholipids of cell membranes; therefore, cell membranes are easily oxidized and lose their functionality. Proteins may also be damaged by ROS, leading to structural changes and loss of enzyme activity. Oxidative damage to DNA also can cause DNA mutation, single strand breaks and chromosomal shortening.[189,195]

2.12.3. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide can be generated through a dismutation reaction from superoxide anion by superoxide dismutase.[207] Monomeric oxidases located in the outer mitochondrial membrane such as amino acid oxidase and xanthine oxidase also produce H_2O_2 from superoxide anion.[185,210] Hydrogen peroxide is highly diffusible and crosses the plasma membrane easily. Once generated H_2O_2 is metabolized by catalase or glutathione peroxidase to generate water and oxygen.[185,210] Although H_2O_2 is the least reactive molecule among reactive oxygen species it is very damaging because it can be converted to hydroxyl radical.[175] In the presence of metals such as Fe^{2+} or Cu^{2+}, H_2O_2 can generate hydroxyl radical through

Fenton type reactions[185,210] in which Fe^{2+} is oxidized to Fe^{3+} and H_2O_2 is converted to $^{\bullet}OH$ and OH^- (equation 11).



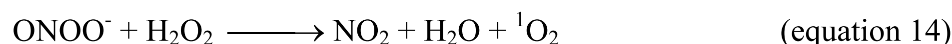
Fenton $$H_2O_2 + Fe^{2+} \longrightarrow {}^-OH + {}^{\bullet}OH + Fe^{3+} \quad \text{(equation 11)}$$

2.12.4. Singlet oxygen

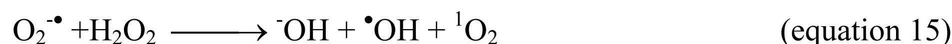
Singlet oxygen is a nonradical oxygen species. Singlet oxygen can be generated from H_2O_2 during interaction with hypochlorite (OCl^-) in cells and tissues (equation 12).[175,180] It can stimulate formation of nitric oxide (NO) and nitric dioxide (ONOO) (equation 13-15). Singlet oxygen is a mild toxin for mammalian tissue in comparison with other reactive oxygen species.[180] However, singlet oxygen has been known to be associated with oxidation of PUFA to initiate lipid peroxidation.[179,207]

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + {}^1O_2 \quad \text{(equation 12)}$$

$${}^-OCl + H_2O_2 \longrightarrow H_2O + Cl^- + {}^1O_2 \quad \text{(equation 13)}$$



$$ONOO^- + H_2O_2 \longrightarrow NO_2 + H_2O + {}^1O_2 \quad \text{(equation 14)}$$



$$O_2^{-\bullet} + H_2O_2 \longrightarrow {}^-OH + {}^{\bullet}OH + {}^1O_2 \quad \text{(equation 15)}$$

2.12.5. Peroxyl and alkoxyl radicals

Peroxyl radicals ($ROO^{\bullet}$) are formed by a direct reaction of oxygen with alkyl radicals ($R^{\bullet}$), for example, the reaction between lipid radicals and oxygen.[197,211] Decomposition of alkyl peroxides (ROOH) also results in peroxyl ($ROO^{\bullet}$) and

alkoxyl ($RO^{\bullet}$) radicals.[184,185,193,208,212] Peroxyl and alkoxyl radicals are good oxidizing agents which are involved in the propagation stage of lipid peroxidation.

2.13. Reactive nitrogen species (RNS)

2.13.1. Nitric oxide and nitric dioxide

Nitric oxide ($NO^{\bullet}$) is formed *in vivo* from L-arginine by Nitric oxide synthase.[181] Nitric oxide itself is not a very reactive free radical, but overproduction of NO can lead to protein oxidation in ischemia/reperfusion and neurodegenerative and chronic inflammatory diseases.[201,208,213] Nitric dioxide ($NO_2^{\bullet}$) is formed from the reaction of peroxyl radical and NO, polluted air and smoking.[214]

2.13.2. Peroxynitrite

Peroxynitrite can be generated by the reaction of $NO^{\bullet}$ and superoxide anion (equation 16). Peroxynitrite is a cytotoxic species and causes tissue injury and oxidizes LDL.[194,215,216] Peroxynitrite ($OONO^{-}$) can cause direct protein oxidation and DNA base oxidation.[204]

$$O_2^{-\bullet} + NO^{\bullet} \longrightarrow ONOO^{-} \qquad \text{(equation 16)}$$

2.14. Protein oxidation

Proteins are highly susceptible to oxidative damage. Modification of proteins is mainly initiated by hydroxyl radicals, leading to the oxidation of amino acid side chains (where sulfur-containing amino acids are found) and protein

fragmentation.[217,218] Malonaldehyde from lipid oxidation can react with protein amino groups to form adducts.[217,219] Peroxynitrite ($ONOO^-$) can oxidize essential –SH groups on proteins.[219] Protein oxidation alters signal transduction mechanisms, transport systems, and enzyme activities, and can lead to atherosclerosis and ischemia reperfusion injury.[220] Aging is associated at least partly with oxidative modification of proteins.[217]

2.14.1. DNA strand breaks

Mitochondrial DNA is susceptible to oxidative damage because of the lack of protective protein and close proximity to the reactive oxygen species-producing systems.[183] Oxidative damage of DNA leads to mutagenesis and carcinogenesis.[183] DNA can undergo oxidative damage at both the nucleic bases (the individual molecules that make up the genetic code) and at the sugars that link the bases.[221] Oxidative damage of DNA results in degradation of the bases, breaking of the DNA strands by oxidation of the sugar linkages, or cross-linking of DNA to protein (a form of damage particularly difficult for the cell to repair). Although all cells have some capability of repairing oxidative damage to proteins and DNA, excess damage can cause mutations or cell death.[172,173]

2.14.2. Nucleic acid oxidation

Nucleic acids are pentose-phosphate polymers that can undergo reactions with hydroxyl radical.[221] The base modifications in DNA nucleic acids may be

responsible for genetic defects produced by oxidative stress. Among the four DNA bases, guanine has the lowest oxidation potential and is most easily oxidized.[221] Recently, 8-hydroxy guanosine has generated considerable interest as a biomarker of oxidative stress that can be used to estimate DNA damage in humans.[222]

Urinary level of modified bases is a useful means of assessing the amount of DNA damage in an animal. Products such as 8-hydroxy guanosine, thymidine glycol, and uric acid are used for these estimates.[221] Formation of 8-oxoguanine induces Guanine:Cytidine to Thymine:Adenine transversions at the DNA replication stage, an important process in carcinogenesis and tumor development.[221-222] DNA damage has also been estimated by chain breaks and base modifications in cultured cells under oxidative stress. An important metabolic effect of DNA damage is the rapid induction of polyadenosine diphosphate ribose synthesis (ADP-ribosylation) in nuclei, resulting in extensive depletion of cellular NADH.[221] ADP-ribosylation has been associated with repair of damaged DNA.

2.15. Lipid Peroxidation in humans

Lipid peroxidation is a self-propagation reaction that leads to generation of lipid radicals and lipid peroxides.[173] Cell membranes are phospholipid bilayers and proteins, which are the direct targets of lipid oxidation.[172,173] As lipid oxidation of cell membranes increases, the polarity of lipid-phase and formation of protein oligomers increase but molecular mobility of lipids, number of SH groups, and resistance to thermodenaturation decrease.[172,173]

During lipid oxidation, malonaldehyde (product of oxidation) can react with the free amino group of proteins, phospholipids, and nucleic acids damaging their structures and functions.[179,185,204,218] Increased levels of lipid oxidation products are associated with diabetes and atherosclerosis.[173,216] Oxidation of low-density LDL has been reported to be involved in the development of atherosclerosis and cardiovascular disease.[170,172,216] Oxidized cholesterol or fatty acid moieties in plasma LDL can lead to the development of atherosclerosis.[173]

2.15.1. Lipid Peroxidation in foods

Lipid peroxidation can cause rancidity of food products such as fats and oils, which affects odor, taste and nutritional value of these products.[223,224] The main purpose of using antioxidants in foods is to delay, retard, or prevent the autoxidation process and thus to extend the shelf life of foodstuffs and to minimize nutritional losses.[223]

Lipid peroxidation (Figure 2.12) involves hydrogen abstraction to form a lipid radical ($L^{\bullet}$) (equation 17), which can react with oxygen to form a lipid peroxyl radical (equation 18). The peroxyl radical propagates the chain reaction by abstracting hydrogen from another lipid (equation 19), usually the rate-limiting step in lipid peroxidation.[224] Ideally, antioxidants work by blocking the propagation step (equation 3) through hydrogen atom donation. Antioxidants can also interfere with the oxidation process by chelating catalytic metals and acting as free radical scavengers.[166]

$R^{\bullet} + LH \longrightarrow RH + L^{\bullet}$ (equation 17)

$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$ (equation 18)

$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$ (equation 19)

Figure 2.12. Lipid peroxidation steps (adapted from Shahidi and Wanasundara).[224]

2.15.2. Antioxidants in food systems-important factors for measuring antioxidant effectiveness

The activities of antioxidants depend not only on their structural features (e.g. activities towards peroxyl and other active species), but also on many other factors, such as concentration, temperature, light, type of substrate, physical state of the system, as well as on microcomponents acting as pro-oxidants or synergists. [225] Several important factors that may affect the results of antioxidant studies in lipid systems are the initial concentration of primary autoxidation products, such as hydroperoxides of the lipid and free fatty acids. Primary oxidation products may reduce both the efficiency of the antioxidants and the time for radical-mediated consumption of antioxidant.[149] Free fatty acids may demonstrate a pro-oxidant effect and should be minimized. Other lipid microcomponents, such as metals, fatty alcohols and mono- and diacylglycerols, have been also shown to decrease the effectiveness of phenolic antioxidants.[149]

Temperatures at which antioxidant activity may be required range from 180–200°C for deep frying oils, to about 5°C for products such as margarine or

mayonnaise that are stored in the fridge.[225] Besides the processing and storage temperatures to which these products are exposed, the accompanying constituents including water, proteins, carbohydrates, vitamins, minerals and other food components vary, and the physical structure of the food also varies. This can cause big changes in the activity of the antioxidant in different food systems.[149] Antioxidants are normally less effective at elevated temperatures than at ambient temperature. Therefore, it can be difficult to predict antioxidant effects at low temperatures from measurements at elevated temperatures.

Antioxidants that are heat stable have the property referred to as 'carry-through'.[225] Antioxidants are divided into two categories, preventive antioxidants and chain-breaking antioxidants.[226] The preventive antioxidants (e.g. glutathione peroxidase and catalase) deactivate the active species (e.g. H_2O_2) without further generation of free radicals and, thereby, reduce the rate of chain initiation.[226] Chain-breaking antioxidants have the ability to scavenge chain-propagating oxygen radicals to produce stable, non-radical products and suppress lipid peroxidation.[226] Therefore, the antioxidant capacity, *n*, shows how many chains may be terminated by one molecule of antioxidant.[226,227] BHT **17** traps peroxyl radicals to produce a stable phenoxyl radical or to form stable non-radical products.[197,228] Niki *et al.* (1985)[228] demonstrated that chain-breaking antioxidants, such as vitamin E, can scavenge the chain-propagating oxygen radicals generated from 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) and suppress the peroxidation of liposomal membranes in aqueous dispersions.[226] Lee *et al.* (2004)[197] reported that cysteine and

glutathione could also act as chain-breaking antioxidants by scavenging oxygen-centered radicals and suppressing the oxidation of lipids.[197]

The antioxidant properties of naturally-occurring catechols have been studied extensively so that many stoichiometric ratios or antioxidant capacities for these catechols have been determined. When inhibition of the oxidation of cumene in methyl linoleate is measured, several catechols demonstrate impressive antioxidant capacities such as quercetin (2.6), (-)-epigallocatechin (2.2), catechin (1.7) and methylgallate (2.3).[227]

There are several important factors that must be considered when attempting to measure or assess the antioxidant effectiveness of a compound in foods.

1) Food model system

One of the major factors affecting the activity of antioxidants that scavenge free radicals in foods is their partitioning behavior in lipids and water. It is commonly observed that a non-polar antioxidant such as (α-tocopherol) is relatively ineffective in oil but is strongly effective in an oil-in-water emulsion. In contrast, a polar antioxidant such as ascorbic acid or trolox (a water-soluble derivative of α-tocopherol) is more effective in an oil than in an emulsion. This phenomenon has been described as the polar paradox.[225]

Antioxidant are most effective when they are at or near the physical location of the oxidative reactions.[149] The increased antioxidant ability of the polar antioxidant in the bulk oil phase is the result of a preference of the antioxidant for the oil surface, where the majority of autoxidation would be expected to occur. An emulsion

consists of two immiscible liquids, with one liquid being dispersed as small diameter (0.1-100 mm) spherical droplets in the other liquid. Food emulsions can exist as oil-in-water emulsions (e.g. milk) where oil is dispersed in water or water-in-oil emulsions (e.g. mayonnaise) where water is dispersed in oil.[149] An emulsion can be considered to consist of three regions: the interior of the droplets, the continuous phase, and the interfacial membrane. The interfacial membrane consists of a narrow region surrounding each emulsion droplet that consists of a mixture of oil, water, and emulsifier molecules.[149] Nonpolar molecules are mainly located in the oil phase and polar molecules are mainly located in the aqueous phase and at the interface. The precise molecular environment of a molecule may have a significant effect on its chemical reactivity. The nature of the emulsion droplet interfacial membrane and the physical location of antioxidants in emulsions are critical to antioxidant effectiveness. Therefore it is important that an appropriate system is chosen for the antioxidant of interest.

2) Effective analytical techniques

It is frequently useful to have antioxidant assays that are capable of measuring both primary (e.g., hydroperoxides, conjugated dienes) and secondary oxidation (carbonyls, volatile compounds) products.[229] In some cases antioxidants (e.g., tocopherols) can increase primary lipid oxidation products by donating hydrogen to a peroxyl radical to form a lipid hydroperoxide while simultaneously decreasing formation of low molecular weight volatile secondary oxidation products. Other compounds can decrease lipid hydroperoxides (e.g., oxidizing

agents such transition metals) while increasing the secondary lipid oxidation products that cause rancidity.[229] For example, if only lipid hydroperoxides are measured to evaluate an antioxidant, an extract of biological material high in iron could appear to be antioxidative because lipid hydroperoxides are being decomposed into the volatile oxidation products that cause rancidity and would never accumulate.[149] It is also possible that nucleophilic compounds can form complexes with aldehydes. Thus, if measuring only a single aldehyde as an index of lipid oxidation, certain compounds could appear to be antioxidative because they could complex that aldehyde, thereby causing its decrease, while not affecting other volatile lipid oxidation components that can affect rancidity.[149]

In vitro assays that measure free radical scavenging activity assays such as the ferric reducing/antioxidant power (FRAP), Trolox-equivalent antioxidant activity (TEAC) and oxygen radical absorbant capacity (ORAC) are performed in the absence of lipids.[149,229] This means that the impact of antioxidant partitioning is not evaluated thus leading to lack of correlation between the results of *in vitro* assays and antioxidant performance in foods. In order to accurately evaluate the potential of antioxidants in foods, models must be developed that have the chemical, physical and environmental (e.g. pH and ionic strength) conditions expected in food products.[149,229] Since these factors are not consistent throughout all food systems, individual models must be developed.[230] Some researchers have recommended using more than one assay for measuring antioxidants in foods so that results can be compared more easily and accurately.[149,229] Assays for measuring antioxidant

activity in food systems may be divided into (a) assays based on primary oxidation products and (b) assays based on secondary oxidation products. Use of a combination of these assays frequently provides more information regarding the potential of the compound as a food preservative.

a) Assays based on primary oxidation products

The peroxide value (PV) method (e.g. ammonium thiocyanate assay) can determine the oxidative stability of unsaturated oil.[230,231] Peroxide value measurements however, are unable to detect any deterioration such as measuring off flavour in foods.[149]

Assays to measure the ability of an antioxidant to inhibit lipid oxidation under accelerated conditions (e.g. rancimat method) have the advantage of being able to simulate a long induction period in a short time.[232] These accelerated test systems mainly include lipids by increasing temperature.[150,232] The Rancimat method measures the amount of volatile hydroperoxides as primary oxidation products.[230] This method suffers from possible changes in the mechanism of oxidation and stability of the antioxidant at elevated temperature.

During lipid oxidation, unconjugated fatty acids can be converted to conjugated fatty acids (conjugated dienes). Therefore, methods have been developed to measure conjugated dienes.[230] These methods artificially induce autoxidation of linoleic acid or LDL by either Cu(II) or an azo initiator (e.g. AAPH or AMVN). The progress of autoxidation is monitored by measuring UV absorbance at 234 nm for the formation of conjugated diene peroxides from linoleic

acid oxidation.[230] This method is suitable provided that decomposition of the primary conjugated diene products does not interfere.

b) Assays based on secondary oxidation products

A portion of the lipid hydroperoxides that form in the early stages of lipid oxidation are broken down to form the low molecular weight volatile compounds (secondary products) that impart rancidity. Various low molecular weight aldehydes, alkenals, and nonvolatile precursors of these substances react with 2-thiobarbituric acid, resulting in chromogens, termed thiobarbituric acid reactive substances (TBARS), that can be determined after acid extraction. Another method for determining secondary oxidation products is to measure volatile oxidation products, typically by headspace analysis using a gas chromatography (GC) technique.[229] Good correlations have been observed between TBARS and headspace volatiles (e.g., hexanal, pentanal) in determination of muscle tissue oxidation.[225,229]

A number of assays exist which measure the ability of an antioxidant to scavenge free radicals. Assays for radical scavenging ability that are commonly used include: measuring the reduction of stable radicals (DPPH radicals), measuring the ability to scavenge radicals generated using azo-initiators (AAPH, AMVN and ABTS), and scavenging of hydroxyl radical generated by the Fenton reaction based radical assay (FRBR assay) or one of several other methods.[150,230,233]

Some general considerations that should be observed for all food products during antioxidant activity measurements are listed as follows:

1. Avoid high oxidation temperatures (>60°C) during storage studies. High temperatures can cause rapid decomposition of hydroperoxides, decomposition or volatility of antioxidants, and a decrease of oxygen solubility.

2. Ensure that the starting lipid does not contain high levels of oxidation products (e.g. transition metals hexanal, free fatty acids, lipid hydroperoxides).[225]

3. When possible, use analytical techniques to measure both primary and secondary oxidation products.[149]

4. The fatty acid composition, degree of unsaturation and position of double bonds for the sample should be known.[229]

5. The use of pure compounds will allow a better evaluation and understanding of how the compound functions alone and in combination with other food components.[229]

6. When antioxidant activity in emulsions is evaluated, it is important to utilize emulsion systems similar to those found in the food of interest.

7. Use the same molar concentration of tested components (e.g., phenolics) as the reference compound (e.g BHT, BHA) which are in the range that would be used in the food product.[225]

8. Use a food lipid with a consistent source (e.g. sampling from one batch) of natural endogenous antioxidants or pro-oxidants for all analyses to minimize variation.[149]

9. pH can affect oxidative reactions by influencing pro-oxidant (e.g., iron solubility increases with decreasing pH) and antioxidant (the pH can alter the charge of

antioxidants, which can affect solubility and chelation capacity) activity. The pH of oxidation models should therefore be similar to the food of interest. [229]

10. Standardize time and conditions to determine if the antioxidant is effective. Appropriate ways to express antioxidant effectiveness include: a difference in induction period, percent inhibition of compound formation at a set time, rate of compound formation or decomposition, IC_{50} (antioxidant concentration to achieve 50% inhibition), or percent loss or retention of antioxidant.[225]

2.15.3. Synthetic antioxidants in foods

The antioxidant activity of phenolic compounds such as butylated hydroxy toluene (BHT) **17** and butylated hydroxy anisole (BHA), tertiary butylhydroquinone (TBHQ), and esters of gallic acid, e.g. propyl gallate (PG), have been well studied.[224] Synthetic phenolic antioxidants are usually used at the level of 0.02 % of the fat or oil content of the food.[225] The disadvantages of gallates is their heat sensitivity and their tendency to form dark precipitates with iron ions. Some antioxidants, such as BHA and BHT, are used in combination with resulting synergistic effects.

In general, phenolic antioxidants such as BHT **17** and BHA appear to trap peroxyl radicals via a two step process: 1) a peroxyl radical abstracts a hydrogen atom from the hydroxyl group, producing a stable phenoxyl radical; 2) the phenoxyl radical reacts with another peroxyl radical to form stable non-radical products (Figure 2.13).

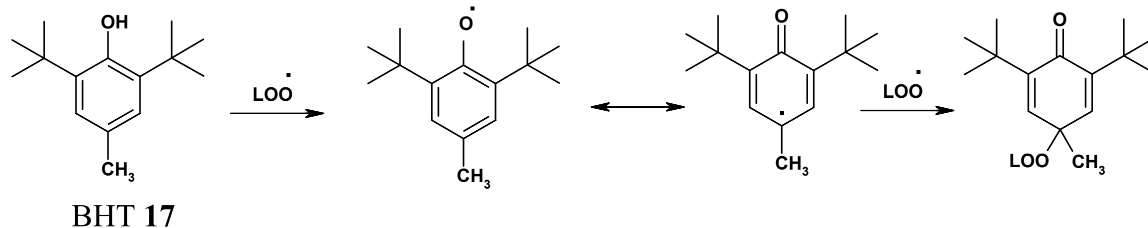


Figure 2.13. Formation of stable non-radical products using antioxidant BHT **17** (adapted from Shahidi and Wanasundara).[224]

2.16. Enzymic detoxification of xenobiotics in the body

The enzymic detoxification of xenobiotics has been classified mainly into Phase I and Phase II detoxification. Phases I and II involve the conversion of lipophilic, non-polar xenobiotics into more water-soluble molecules, which can then be eliminated more easily from the cell. Phase I processes are catalysed mainly by the cytochrome P-450 (CYP450) system.[234] This family of microsomal proteins is responsible for a range of reactions, of which oxidation appears to be the most important.[235] The major CYP450 enzymes involved in metabolism of drugs or exogenous toxins are the CYP3A4, CYP1A1, CYP1A2, CYP2D6, CYP2C and CYP2E1 enzymes.[236]

Phase II conjugation reactions generally follow Phase I metabolism, resulting in a xenobiotic that has been transformed into a water-soluble compound that can be excreted through urine or bile.[236] Several types of conjugation reactions are present in the body, including glucuronidation, sulfation, and glutathione and amino acid conjugation.[181,234] These reactions require cofactors, which must be provided through dietary sources. Quantitatively, conjugation to glutathione (GSH), which is

catalysed by the Glutathione S-transferases (GSTs), is the major phase II reaction in many species.[237] GSTs also can catalyse the reduction of hydroperoxides, resulting in the formation of oxidized glutathione (GSSG).[237] Some of important detoxification enzymes in the body are explained in the following sections.

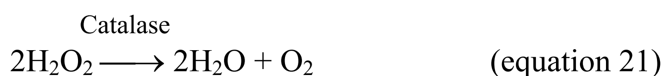
2.16.1. Superoxide dismutase (SOD)

SOD is present in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme) in order to maintain a low concentration of superoxide anion.[178,234] It catalyzes the dismutation of superoxide anion into oxygen and hydrogen peroxide according to the following reaction (equation 20):

$$2O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{(equation 20)}$$

2.16.2. Catalase

Catalase is a heme protein that catalyses the detoxification of hydrogen peroxide (equation 21).[172] Catalase provides a protective role that is similar to that of glutathione peroxidase because both are important means of removing hydrogen peroxide.[205]



$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \qquad \text{(equation 21)}$$

2.16.3. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) is a cytoplasmic and mitochondrial enzyme that is important for detoxifying peroxides in the cell.[234] The GPx enzymes catalyze the reduction of H_2O_2 to water and organic peroxides (R-O-O-

H) to the corresponding stable alcohols (R-O-H) using glutathione (GSH) as a reducing source (equation 22).[234]

$$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O \qquad \text{(equation 22)}$$

2.16.4. Glutathione (GSH)

Glutathione (GSH, γ-glutamylcysteinylglycine), the primary non-protein sulfhydryl enzyme in aerobic organisms, is synthesized in most cells.[210] The tripeptide is formed by the combination of glutamic acid and cysteine, catalyzed by γ-glutamylcysteinyl synthetase. Glycine is then added by glutathione synthetase to form GSH.[210] The sulfur atom in cysteine is able to partially ionize at neutral pH, i.e., the pH found in normal cells. This produces a different reactivity than the chemistry associated with a sulfhydryl group. Therefore in the case of glutathione there are two forms that differ only in the presence or absence of the proton on the cysteine moiety of the peptide (equation 23).[210] The anionic form of glutathione is a strong nucleophile. Since there is very little of the anion at pH=7, cells have developed a family of enzymes called glutathione S-transferases that make glutathione a more reactive nucleophile.[181,234]

$$GSH \longrightarrow GS^{-} + H^{+} \qquad \text{(equation 23)}$$

2.16.5. Glutathione S-transferases (GSTs)

GSTs are a large group of multifunctional proteins that catalyse the conjugation of GSH to various electrophilic substrates.[210] GSTs appear to play an important role in protecting cells against oxidative damage by: 1) binding glutathione in such a way that the sulfur is induced to ionize more completely, and 2) binding a second molecule close by so that a reaction can be facilitated.[181,234] This reaction is necessary to detoxify xenobiotic materials such as toxins, drugs, and other foreign compounds.[210]

2.16.6. Glutathione reductase

The flavoprotein, glutathione reductase, uses the reducing power for the pentose phosphate pathway (NADPH) to keep the glutathione pool in a cell in a very reduced state (equation 24).[210] Cells contain at least 100 reduced glutathione molecules for every molecule of glutathione disulfide.[182]

$$\mathrm{GSSG + NADPH \longrightarrow 2GSH + NADP^{+} + H^{+}} \qquad \text{(equation 24)}$$

The net result of this cycle is to use NADPH to reduce hydrogen peroxide to water, a process that requires two electrons.[182] Other reductases can also catalyze reactions that reduce lipid peroxides, i.e., LOOH, instead of hydrogen peroxide (equation 25).

$$\mathrm{LOOH + 2GSH \longrightarrow GSSG + LOH} \qquad \text{(equation 25)}$$

2.17. Non-enzymic detoxification of xenobiotics in the body

2.17.1. Tocopherols

Tocopherols (i.e.Vit E) are natural constituents of biological membranes. Antioxidant mechanisms of tocopherols include the transfer of a hydrogen atom from the 6–hydroxyl group on the chroman ring, and scavenging of singlet oxygen and other reactive species.[215,228] Tocopherols are regenerated in the presence of ascorbic acids.[215,228] Tocopherols can protect PUFA within the membrane and LDL, and inhibit smooth muscle cell proliferation.[238,239] Tocopherol has been associated with the reduction of heart disease, delay of Alzheimer's disease, and prevention of cancer.[238] Tocopherols have beneficial effects in cardiovascular diseases both by inhibiting LDL oxidation and by down-regulating hydroxy-methylglutaric CoA reductase (HMG-CoA reductase), a key enzyme of the mevalonate pathway.[238,239]

2.17.2. Ascorbic acid

The antioxidant mechanisms of ascorbic acid are based on hydrogen atom donation to lipid radicals, quenching of singlet oxygen, and removal of molecular oxygen.[240] Ascorbic acids help to regenerate tocopherols by donating a hydrogen atom to a tocopheroxyl radical. Ascorbic acid and tocopherol supplementation can substantially reduce oxidative damage.[240]

2.18. Plant-derived antioxidants

2.18.1. Flavonoids

Flavonoids **18** (Figure 2.14) are polyphenolic compounds that are abundant in fruits, vegetables, red wine, tea and chocolate.[195,241-243] They display a multitude of biological effects *in vitro* and *in vivo* after consumption of flavonoid-containing foods. Epidemiologic studies show that increased consumption of flavonoids reduces the risk of cardiovascular disease and certain types of cancer.[148,244,148,244] Flavonoids may exhibit antioxidant, antimutagenic, and free-radical scavenging activities.[245] Moderate consumption of red wine, which contains a high content of polyphenols, is associated with a low risk of coronary heart disease.[246,247] Consumption of soy and soy products are related to biological effects, including anticarcinogenic, antiatherosclerotic, and antihemolytic effects.[245,247] The daily average consumption of flavonoids as 1 gram showed higher antioxidant activity than ascorbic acid and α-tocopherol.[247]

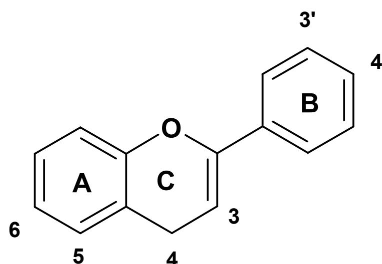


Figure 2.14. Structure of flavonoid **18** (adapted from Lee *et al.,* 2004).[197]

Flavonoids **18** generally consist of two benzene rings (rings A and B) linked by an oxygen-containing heterocycle (ring C) (Figure 14).[197] There are six classes of flavonoids including: flavanones, flavones, flavonols (catechins and proanthocyanidins), isoflavonoids, anthocyanins, and flavans, which vary in structure. The antioxidant properties of flavonoids have been well studied and are structure-dependent with the major contributing factor being the catechol structure at ring B (two adjacent hydroxyl groups at 3′,4′-positions) (Figure 2.14). Hydroxyl substituents on the flavonoid ring increase the antioxidant activity, while substitution by methoxy groups decrease this activity.[248,249] Many of the techniques used to study flavonoid antioxidant properties are relevant to the study of lignan antioxidant properties. Understanding the antioxidant properties of flavonoids may provide some insight into the antioxidant properties of lignans.

2.18.3. Flavonol

2.18.3.1. Quercetin

The catechol **19** (Figure 2.15) containing flavonoid structures [quercetin **20**, catechin **26** and (-)-Epicatechin **28**] are well known to be H-donating antioxidants. In Figure 2.16, the structure of quercetin **20** and the mechanism of hydrogen donation during oxidation of quercetin are depicted. Interestingly, the hydroxy groups on the chromane-like flavonoid of quercetin **20** do not appear to participate directly in oxidation. Instead, it is the hydroxy groups of the catechol moiety, the B ring, that donate or accept hydrogens.[250-252] *In vitro* incubation of quercetin in

normal human plasma and *in vivo* study of rats fed with a quercetin-enriched diet showed that quercetin is extensively bound to plasma proteins.[253]

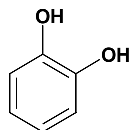
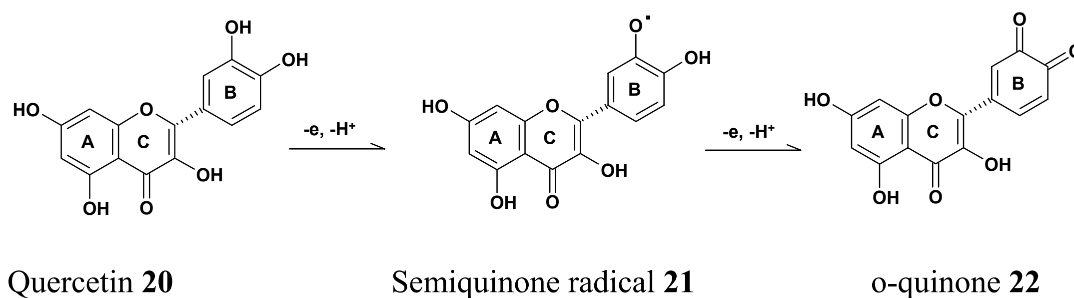


Figure 2.15. Structure of catechol **19**.[197]



Quercetin **20** Semiquinone radical **21** o-quinone **22**

Figure 2.16. Oxidation of quercetin **20**.[197]

2.18.3.2. Catechins

Flavanols **29** (Figure 2.17) exist in both the monomer form (catechins) and the polymer form (proanthocyanidins).[197,248] Green tea and chocolate are by far the richest sources of catechin **26** contaning food.[197,248] Catechins are also present in red wine.[197,248] In contrast to other classes of flavonoids, flavanols are not glycosylated in foods.[197,248]

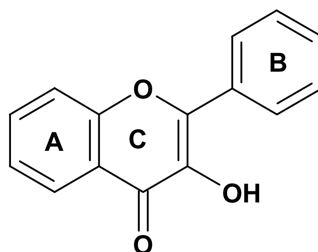


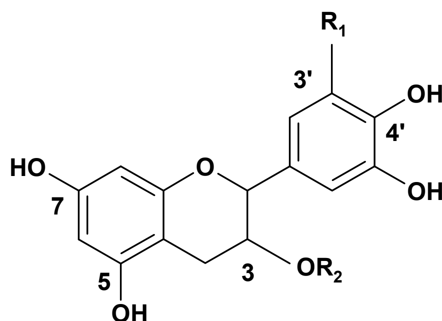
Figure 2.17. Structure of flavonol **29** (adapted from Lee *et al.,* 2004).[197]

Catechins have antioxidant and inhibitory activity against tumors.[242,244,254] The green tea catechins make up approximately 30% of the dry weight of green tea leaves. Black tea contains fewer monomer flavanols, which are oxidized during fermentation of tea leaves to the major pigments that are more complex condensed polyphenols known as theaflavins (dimers) and thearubigins (polymers).[242,244,254]

Epidemiologic studies suggest that both green tea and black tea consumption may reduce the risk of cardiovascular diseases.[242] *In vitro* studies showed that the antioxidant activity of catechins at 40 μM were comparable to the commercial antioxidants BHT **17** and vitamin E at 10 μM.[255] They also are capable of preventing DNA oxidation particularly in the presence of transition metal ions.[256] Methylation of tea catechins also may protect human fibroblasts from oxidative-stress-induced cell death.[257] Catechins have the ability to induce apoptosis cancer cells.[258] Catechins have been shown to inhibit COX enzymes *in vitro*, which a modulation of the inflammatory process.[258]

There are four major catechins in tea: (-)-Epigallocatechin (EGC) **27**, (-)-Epicatechin (EC) **28**, (-)-Epigallocatechin-3-gallate (EGCG) **29**, and (-)-Epicatechin-

3-gallate (ECG) **30**.[242,259] Of the catechins, EGCG is the most abundant one in green tea leaves (Figure 2.18).



Tea polyphenols	R_1	R_2
(-)-Epicatechin (EC)	-H	-H
(+)-Epicatechin (EC)	-H	-H
(-)-Epigallocatechin (EGC)	-OH	-H
(-)-Epicatechin gallate (ECG)	-H	—CO— (OH, OH, OH)
(-)-Epigallocatechin gallate (EGCG)	-OH	—CO— (OH, OH, OH)

Figure 2.18. Chemical structure of tea polyphenols.[197]

O-methylation may be one of the major metabolic pathways for catechin metabolism in mammals.[244,259] Figure 2.19 illustrates formation of tea catechin metabolites. Most of these compounds exist in the glucuronide and sulfate conjugated forms.[244,259]

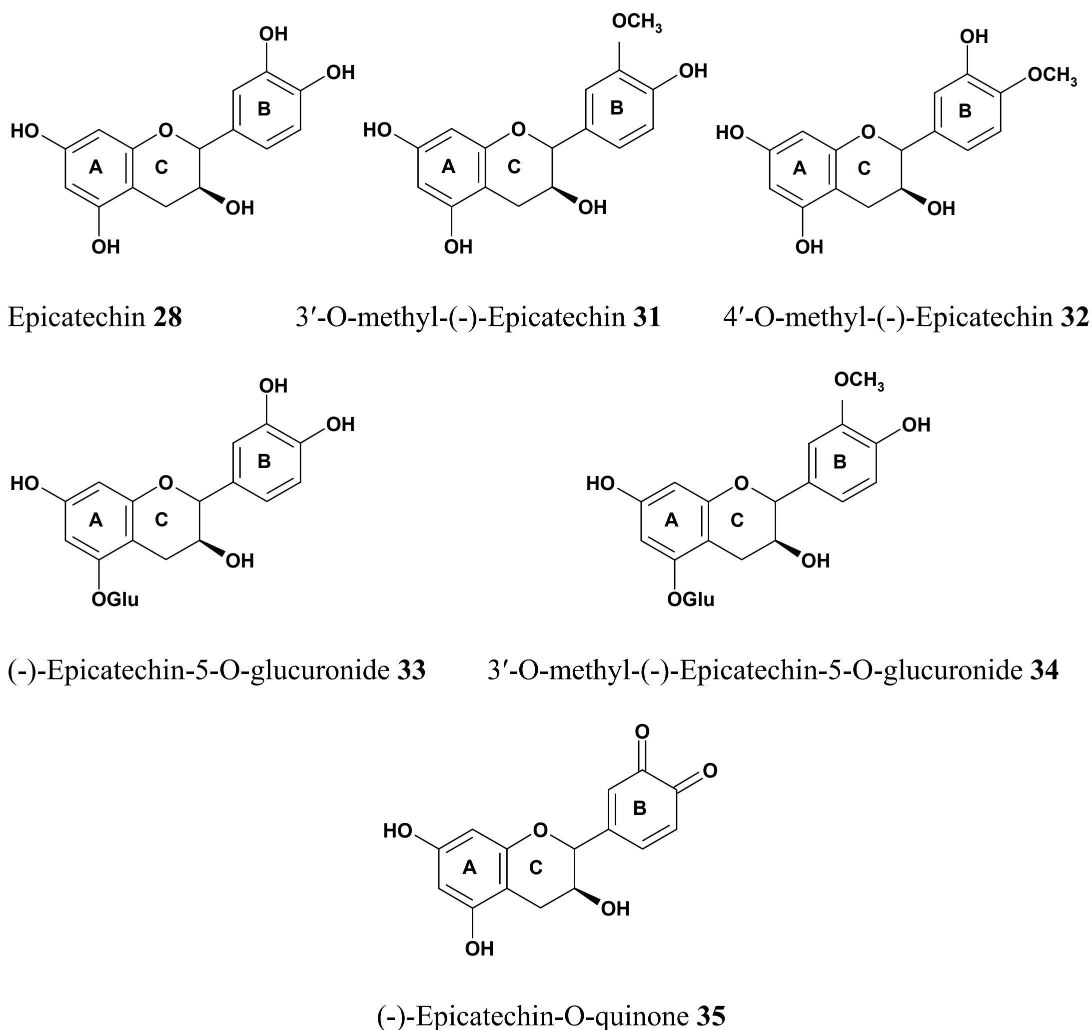


Figure 2.19. Structures of (-)-Epicatechin **28** metabolites: **31** 3′-O-methyl (-) Epicatechin; **32** 4′-O-methyl-(-)-Epicatechin; **33** (-)-Epicatechin-5-O-glucuronide; **34** 3′-O-methyl-(-)-Epicatechin-5-O-glucuronide; **35** (-) Epicatechin- O-quinone.[197]

Dimer formation occurs following oxidation of epicatechin **28** (Figure 2.20). A remarkable feature of dimers of this kind is that they contain the same number of active OH groups on a monomer unit basis as the original compound.[227]

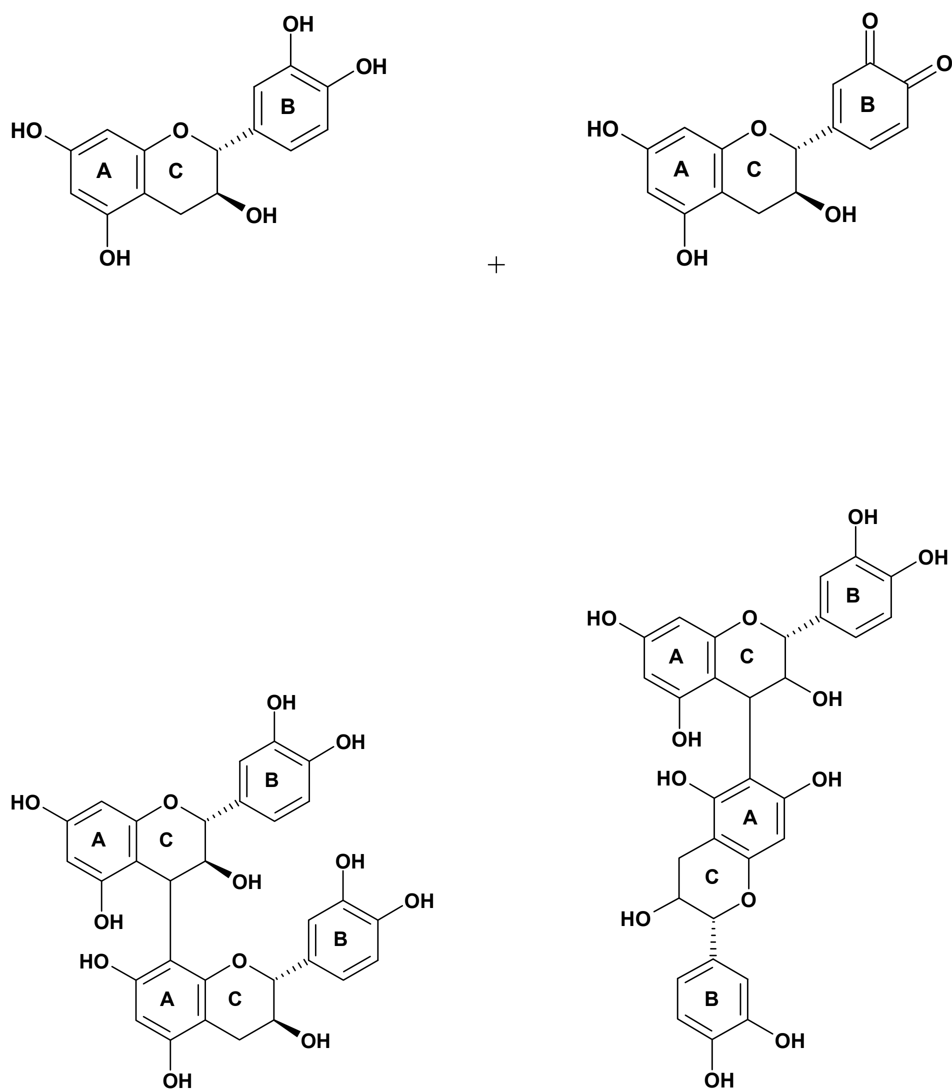


Figure 2.20. Formation of dimers from Epicatechin **28**.[227]

The infuence of methylation of the catechol **19** group on the antioxidant activity of epicatechin, in the form of 3′-*O*-methyl epicatechin **31**, was determined using the TEAC assay.[227] Such assays are designed for antioxidant capacity of antioxidants through their hydrogen-donating abilities. The TEAC values of

epicatechin and 3′-*O*-methyl epicatechin (relative to Trolox) were 4.8 and 1.8, respectively, indicating that the H-donating ability is greatly reduced on methylation of the B-ring.[227,197]

The partitioning of polyphenols and their metabolites between aqueous and lipid phases is largely in favor of the aqueous phase because of their hydrophilicity. Partitioning of polyphenols for binding to albumin was 99% (up to 15 μmol/L), whereas binding to VLDL was not significant (<0.5%).[253] 3′-*O*-methyl epicatechin **31** would be expected to enter cells more freely than epicatechin **28** itself due to its higher lipophilicity.[227] It was suggested that health benefits of flavonoids such as epicatechin **28** is not only due to the native compound but also by the ability of their metabolites to interact with cell-signalling cascades, to infuence the cell at a transcriptional level and to down-regulate pathways leading to cell death.[257,259]

2.18.3.3. Proanthocyanins

Proanthocyanidins, which are also known as condensed tannins, are dimers, oligomers, and polymers of of catechins.[148] They are made up of EC **28** or EGCG **29** that are known as procyanidins **36** or prodelphinidins, respectively.[148,197] Figure 2.21 illustrates the trimeric form of anthocyanidins.

In France, coronary heart disease mortality is low despite a high intake of saturated fats and relatively high plasma cholesterol levels; this is called the "French paradox."[148,197] High consumption of wine was found to be related with the French paradox.[197,260] Tannins and proanthocyanins are the major polyphenols found in

wine.[260] Other phenolic compounds in wine are *p*–coumaric, cinnamic, caffeic, gentisitc, ferulic, and vanillic acids.[197]

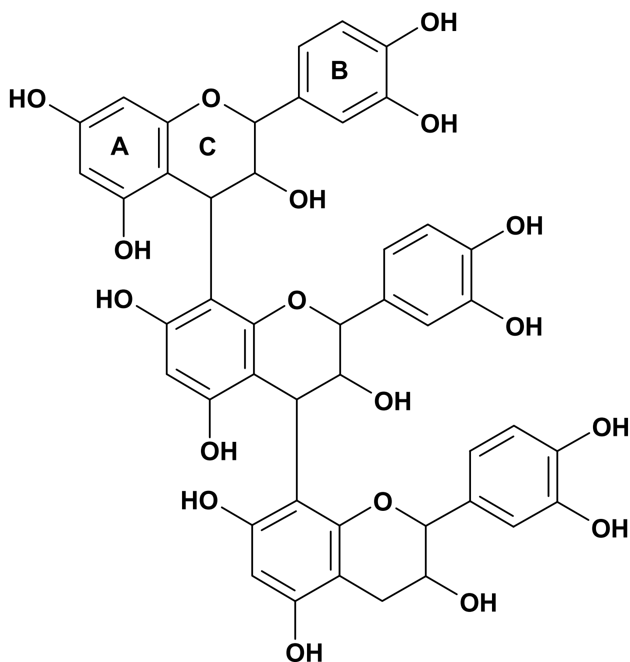


Figure 2.21. Chemical structure of procyanidins **36**.[148,197]

2.18.4. Mechanism of action

In general, mechanisms of flavonoid antioxidant action can include (1) suppressing reactive oxygen and nitrogen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production; (2) scavenging reactive oxygen and nitrogen species; and (3) upregulating or protecting antioxidant defences.[148,226,248] For example, catechins inhibit the enzymes

responsible for superoxide anion production, such as xanthine oxidase and protein kinase C.[148,226,248]

It is believed that antioxidant mechanisms of green tea catechin compounds are based on hydrogen donating abilities and chelating metal ions, which may initiate lipid oxidation.[226] After donating a hydrogen atom, these phenolic compounds become resonance-stabilized radicals, which do not easily participate in other radical reactions. Antioxidant activities of flavonoids are influenced by hydroxylation and the presence of sugar moieties.[247] Flavonoids are effective hydroxyl radical and peroxyl radical scavengers.

As free radical scavengers, flavonoids inhibit lipid peroxidation, promote vascular relaxation and help prevent atherosclerosis.[197] Both animals and humans have shown that increasing polyphenol intake can protect LDL cholesterol from becoming oxidized (a key step in developing atherosclerosis), lower blood pressure in hypertensive subjects, reduce the tendency of the blood to clot and elevate total antioxidant capacity of the blood.[197]

One of the antioxidant properties of green tea catechins is their ability to inhibit oxidation of linoleic acid (an ω-6 fatty acid) from sources such as margarine, safflower oil and corn oil.[197] Excess linoleic acid, most likely in the peroxidized form, can activate various pro-inflammatory enzymes, which are involved in promoting inflammation and tumor growth.[197] When polyunsaturated cooking oils are heated, carcinogenic peroxides can result. It was discovered that catechins act synergistically with α-tocopherol (Vitamin E) to protect against oxidative damage by

oxidized linoleic acid before this fatty acid is incorporated into cell membranes.[148,197]

Catechins have the ability to inhibit peroxynitrite free radicals.[248] Peroxynitrite destroys proteins, as well as endogenous antioxidants such as glutathione and Vitamin E. Green tea catechins are more effective than Trolox in protecting critical amino acids such as tyrosine and the amino acids of apolipoprotein B in LDL cholesterol against nitration damage by the peroxynitrite radical.[227,261] Epicatechin in green tea appears to be metabolized to an anthocyanin-like compound that is also an antioxidant, offering particularly long-lasting protection.

A comparison of the antioxidant properties of simple catechins in green tea versus the complex polymeric polyphenols in black tea found that in lipids the simple catechins were more effective, while in aqueous conditions the polymers had more activity, but glycosylation decreased the antioxidant properties.[241]

Tea catechins play an important role in the inhibition of DNA damage.[254] Tea catechins enhanced the urinary excretion of hydroxylated metabolites via glucuronidation, therefore, it may lower the formation of DNA adducts.[262] UDP-glucuronosyltransferases (UGTs) are increased by green and black tea intake, a detoxification pathway for carcinogen heterocyclic arylamines formed during the cooking of meat, poultry and fish.[262]

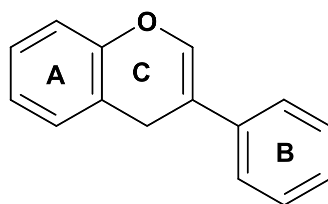
2.18.5. Isoflavones

2.18.5.1 Genistein

Soybeans contain significantly levels of isoflavone **37** (Figure 2.22) levels including genistein **38** and daidzein **39** (Figure 2.23), and their glycosides.[197] Antioxidant activities of isoflavones **37**, especially genistein **38**, were reported *in vivo* and *in vitro*, in simple lipid system such as liposomes, and in more complex system such as lipoproteins.[197,247] Addition of purified forms of isoflavones inhibited copper-dependent LDL oxidation. Oral intake of the isoflavone genistein is associated with decreased LDL and plasma lipid oxidation.[148]

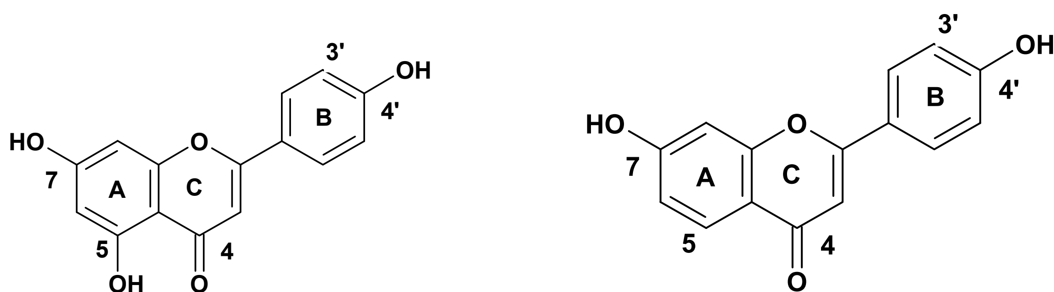
In an oxidation model induced by UVB irradiation, Genistein **38** substantially inhibited a series of oxidative events, including H_2O_2 production, lipid peroxidation, and 8-hydroxy-2-deoxyguanosine formation, an important process in carcinogenesis and tumor development.[63,263,264] Genistein was also studied in a LDL oxidation model initiated by copper and peroxy radicals and was found to inhibit LDL oxidation.[197,263,265-267]

In vitro oxidation of genistein with AMVN-derived peroxyl radicals yielded a hydroxylated derivative of genistein **38** and stable adducts of 4′-oxogenistein with AMVN.[265] This adduct formation occurred on the B-ring of the molecule. In Figure 2.24, genistein **38** oxidation products are illustrated.



isoflavone **37**

Figure 2.22. Structure of isoflavone **37** (adapted from Lee *et al.,* 2004).[197]



38 **39**

Figure 2.23. Structure of genistein **38** and daidzein **39** (adapted from Arora *et al.,* 2000).[265]

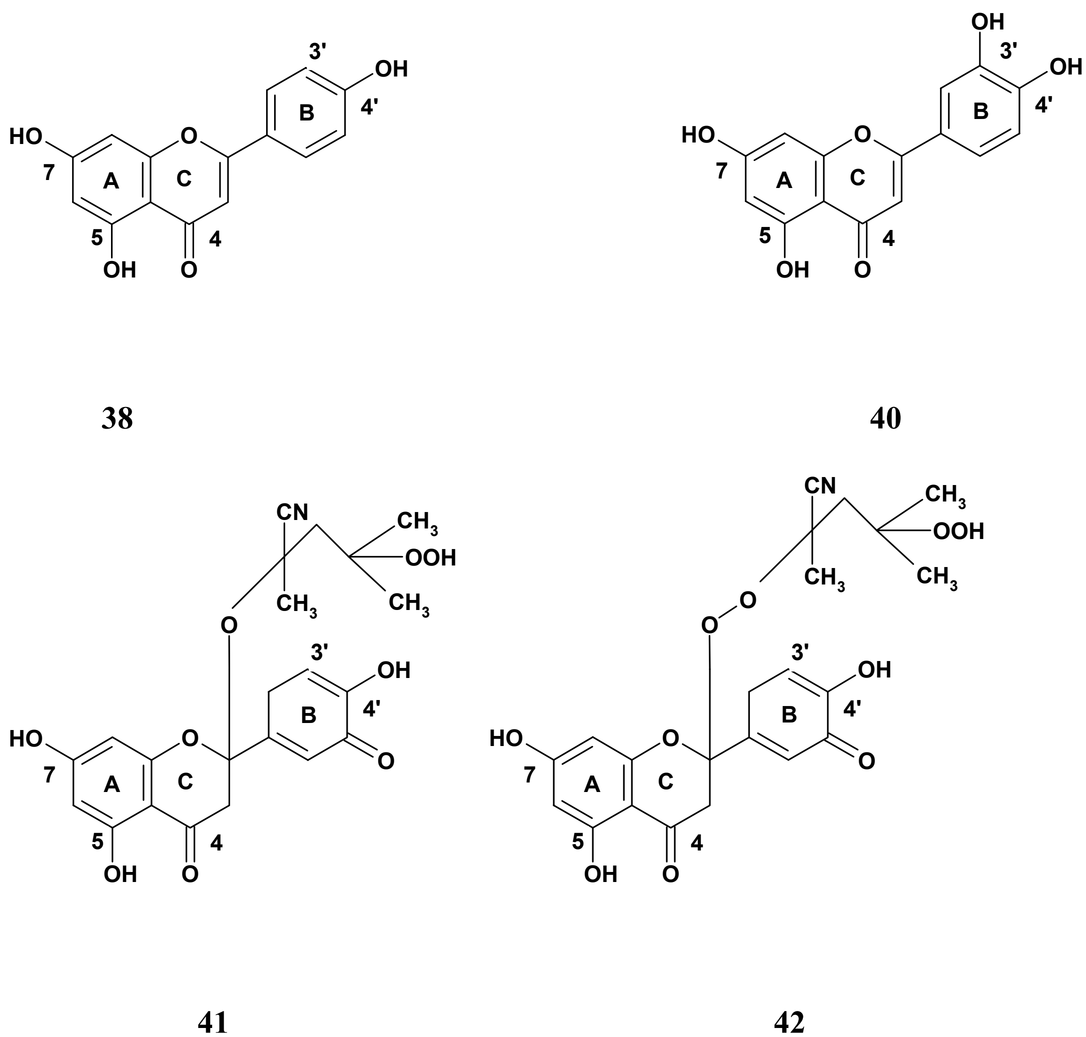


Figure 2.24. Structure of genistein **38** and its oxidation products including: hydroxylated metabolite **40** and 4′-oxogenistein **41** and 4 with AMVN adduct (adapted from Arora, *et al.,* 2000).[265]

2.18.6. Isoflavones-Mechanism of action

The observed health protective actions of soybean isoflavones are mediated by a wide range of mechanisms of which antioxidant activity is probably the most important.[265] Although genistein and daidzein have the same B-ring structure (Figure 2.23), because genistein **38** has one more hydroxyl group than daidzein **39** on the AC-ring, genistein **38** might have higher free radical scavenging capacity than daidzein **39**.[248]

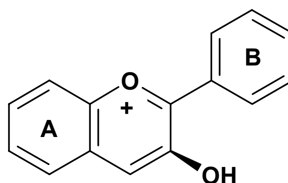
The cholesterol oxidation model results indicated that isoflavone aglycones might have higher antioxidant activities than their glycosides.[226] This would not be important *in vivo* since most of the glycosides would be cleaved to form aglycones by intestinal microorganisms during their absorption in the GI system but could have significance in cholesterol containing foods.[248]

Isoflavones chemopreventive action may arise from their promotion of endogenous antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase.[197] Isoflavones could act at either the transcription level or the protein level to influence the activities of endogenous antioxidant enzymes, but the specific mechanism has not been identified.[197]

2.18.7. Anthocyanidin

Anthocyanidins **43** (Figure 2.25) are major pigments of flowers and fruits that exist in glycoside forms (anthocyanins) in plants.[242] They impart a pink, red, blue, or purple color in plants.[242] They are highly unstable and degradation is

prevented by glycosylation, generally with a glucose at position 3, and esterification with various organic acids (citric and malic acids) and phenolic acids.[242] Cyanidin such as cyanidin 3-galactoside in the skin of fruits is the most common anthocyanidin in foods. Oral administration of red wine polyphenol decreased blood pressure in rats.[242] Moderate red wine consumption may protect against cardiovascular and coronary artery disease.[242,268]

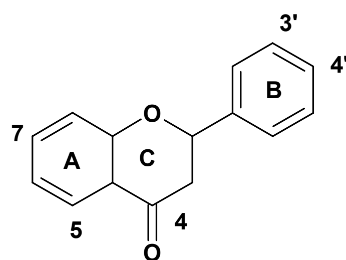


43

Figure 2.25. Structure of anthocyanidin **43** (adapted from Lee *et al.,* 2004).[197]

2.18.8. Flavanones

In human foods, flavanones **44** (Figure 2.26) are found in tomatoes and certain aromatic plants such as mint, but they are high in citrus fruit.[242] Flavanones are generally glycosylated by a disaccharide at position 7. The aglycones forms are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons.

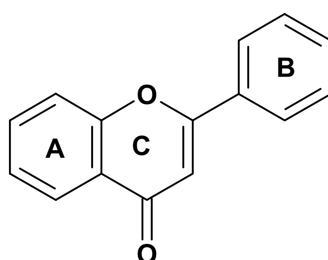


44

Figure 2.26. Structure of flavanone **44** (adapted from Lee *et al.,* 2004).[197]

2.18.9. Flavone

Flavones **45** (Figure 2.27) are much less common than flavonols in fruit and vegetables. The only important edible sources of flavones identified to date are parsley and celery apigenin.[248] The skin of citrus fruit contains polymethoxylated flavones, which can be found in tangeretin and essential oil of mandarin. These polymethoxylated flavones are the most hydrophobic flavonoids.[248]

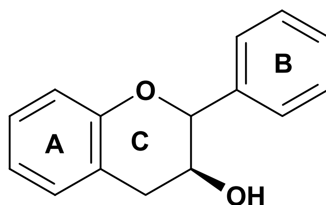


45

Figure 2.27. Structure of flavone **45** (adapted from Lee *et al.,* 2004).[197]

2.18.10. Flavan

The oligomeric proanthocyanidins are flavan **46** (Figure 2.28), usually in the form of flavan-3-ol oligomers, which give anthocyanidins by cleavage of a C–C bond under strongly acidic conditions.[242] The oligomeric proanthocyanidins usually are generated from a flavan-4-ol or a flavan-3,4.



46

Figure 2.28. Structure of flavan **46** (adapted from Lee *et al.,* 2004).[197]

2.19. Potential applications of natural antioxidants in foods

The potential applications for natural antioxidants in foods is growing as consumers become more aware of their potential health benefits.[269] Thus, the market for natural antioxidants has been pushed food makers to source natural preservatives (e.g. rosemary). Little information is available about natural antioxidant as food preservatives and some important factors regarding this are listed as follows:

1) Many natural antioxidants can support structure/function claims but few, if any, can support health claims.[270]

2) The Generally Recognized as Safe (GRAS) regulatory level of approved antioxidants is not high enough to protect product life through manufacturing and distribution.[271]

3) Natural antioxidants are more expensive (10 or 20 times) than synthesized antioxidants on a mass scale.[269]

4) They need an FDA approved standard.[271]

It's taken decades for vitamins C and E to ingrain themselves into the consumer interests.[270] Vitamin E and vitamin C are widely used in oil and oil-based foods. Unlike some of the synthetics, vitamin E can withstand high-heat processes.[271] Recently, rosemary is also labelled, mainly in meat and meat-based foods as natural antioxidant. Food industries intend to provide natural powerful grades for new food applications in order to replace the synthetic antioxidants available in the market.[270] Danisco was the first company that declare the content of rosemary (500-1000 PPM) in their meat products description.[271]

Based on Codex regulation for food antioxidant, BHA, BHT and propyl gallate can be added to raw meat (e.g. beef patties) or cooked meat (e.g. cooked meatballs) at 0.01% of the fat content. They can also be used at 0.02% in combination.[272] This allows levels of BHA and BHT only up to 200 ppm based on the food's fat content.[271,272] A rosemary extract at 1000 ppm, is able to exceed the stability that you would get in a poultry fat at 200 PPM of BHA or BHT.[271,272] For

example, rosemary extract is added at 500 to 1000 ppm in precooked and frozen chicken patties.[270] The advantage of rosemary extracts is that it is more thermally stable than most synthetics.[269,273] In addition to the antioxidative functionality, rosemary extract also has some antimicrobial activity (to kill gram-positive pathogens like *Listeria monocytogenes*).[271]

The natural antioxidant markets will be increased as consumers become more educated about the importance of antioxidants in maintaining and enhancing the quality of life. However, more research that can point specifically to the safety and efficacy of each antioxidant will be needed to make this happen.

2.20. Hypothesis and Objectives

1) The antioxidant stoichiometry of the flaxseed lignans SDG **1** and SECO **2** can be determined in an *in vitro* lipid peroxidation system.
2) The flaxseed lignans SDG **1**, SECO **2** and SDG Polymer **3** can prevent autoxidation in a bulk oil model.
3) The AAPH **47**-mediated antioxidant reactions of the flaxseed lignans SDG **1** and SECO **2** can be determined *in vitro*.

Flaxseed lignans are known to have a number of health benefits, including decreased tumor growth, reduction of serum cholesterol levels and decreased formation of breast, prostate and colon cancers. It is believed that these health benefits of SECO **2** and SDG **1** may be attributed to their antioxidant properties, estrogenic properties, some other mechanism or some combination of all of these.

There is a growing interest in finding alternative antioxidant food preservatives. These include natural products that have the potential to provide health benefits such as the reduction of tumor growth and the lowering of serum cholesterol levels.[6,124,127,151,274] Antioxidant food preservatives such as BHT **17** are commonly used to minimize autoxidation of oil-based food products.[223,275] Some concerns regarding potential liver toxicity from chronic use of these compounds have been expressed.[276] Flaxseed lignans have potential advantages over BHT **17** in that they are natural antioxidants with no known toxicity and thus may also have potential health benefits associated with their consumption.[129,153,277] Little is known about SDG **1** and SECO **2** antioxidant mechanism *in vitro*.

The principal objectives of this study were:

1) Determine the antioxidant stoichiometric ratio for SECO **2** and SDG **1** in liposomes;
2) Determine the ability of SECO **2** and SDG **1** to act as a preservative in food;
3) Determine the products of SECO **2** and SDG **1** antioxidant reactions, using a peroxyl radical system to model lipid peroxidation *in vitro*.

Specific aim for objective (1) was:

- Determine the antioxidant stoichiometric ratio for SECO **2** and SDG **1** in a liposomal suspension using BHT **17** as a standard.

specific aim for objective (2) was:

- Determine the ability of SECO **2** and SDG **1** to prevent lipid peroxidation in vegetable oils using BHT **17** as a standard.

Specific aims for Objectives (3) were:

- Oxidize flaxseed lignans using an *in vitro* model of lipid peroxidation, 2,2′ Azobis-(2-Amidopropane) (AAPH **47**);
- Isolate & purify SECO **2** and SDG **1** oxidation products (using analytical HPLC, semi-prep HPLC, and prep-HPLC);
- Determine structure of oxidation products (using ES-MS and NMR)

3.0. MATERIALS & METHODS

3.1. Materials and solvents

SECO **2,** SDG **1** and SDG polymer **3** were a kind gift from Agriculture & Agri-Food Canada, Saskatoon Research Centre. SECO **2** and SDG **1** were ≥ 99% pure by high performance liquid chromatography (HPLC) and are present as the (R,R) (+)-enantiomer as determined by optical rotation using a Jasco P-1010 polarimeter (Glass cell, length:100mm) (Jasco Corp. Tokyo, Japan). The optical rotation was determined to be: SECO **2** $[\alpha]^{22}_{D}$ = + 35 (*c* 0.20, CH_3OH) and SDG **1** $[\alpha]^{22}_{D}$ = + 0.02 (*c* 0.20, CH_3OH). 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Polyscience Inc. (Warrington, PA). Hydrogen peroxide (H_2O_2, 1%), ammonium thiocyanate and ferrous chloride were obtained from Sigma Chem. Co. (St. Louis, MO). Canola oil (Canola Harvest®) manufactured by Canbra Foods Ltd. (Lethbridge, AB), was purchased from a commercial source (Casco) in Saskatoon. The oil was %100 pure and free of added antioxidants or preservatives. BHT **17** was purched from Sigma (St Louis, MO, USA) and 1,2-Dilinoleoyl-sn-Glycero-3-phosphocholine (DLPC) was from Avanti polar-lipids Inc. (Alabaster, AL). All reagents were HPLC grade. Water was purified using a Millipore super Q water system with one carbon cartridge followed by two ion exchange cartridges (Millipore, USA).

3.2. Experimental 1: Stoichiometry ratio of SECO 2 and SDG 1

3.2.1. Stoichiometry (*n*-value) measurement

Antioxidant stoichiometry was measured using a modification of the method of Liebler and Burr (1995).[278] A liposomal oxidation mixture was prepared using BHT **17**, AAPH **47** and DLPC. The stoichiometric ratio of SECO **2** and SDG **1** was measured by comparison of the lag time for conjugated diene formation (at 240 nm), using UV/VIS spectrophotometer (Agilent 8453, Canada with Agilent Chem Station software) in the presence of SECO **2** and SDG **1**, versus the lag time for conjugated diene formation in the presence of a standard antioxidant (BHT **17**). The stoichiometric ratio (n) of radical trapping was calculated using a kinetic equation (equation 26) described by Liebler and Burr (1995)[278] where Ri (mM min^{-1}) is the rate of chain initiation, [ArOH] is the concentration of the antioxidant, and τ is the length of induction time (min).

$$n = Ri\ \tau/[\mathrm{ArOH}] \qquad \text{(equation 26)}$$

The azo compound AAPH **47** was used to generate peroxyl radicals at a constant rate for each antioxidant. The oxidizable substrate used was DLPC (in chloroform) liposomes. Substrate oxidation was monitored by measuring the formation of conjugated diene at 240 nm, using a spectrophotometer. BHT **17** was used as the antioxidant standard to determine Ri values for autoxidation initiated at 50°C by AAPH **47** peroxyl radicals in Tris-HCl buffer solution containing DLPC

liposomes. The *Ri* values then were used to estimate stoichiometric ratio (*n*) for each antioxidant.

Liposomes were prepared following a modification of Azuma *et al* (1999).[275] Briefly, BHT **17** (in hexane) at two different concentrations (0.53 mM and 0.80 mM) was added to a screw-capped wide mouth 5mL vial, and the hexane evaporated under nitrogen while rotating the vial. In the same vial, 5 mg DLPC (in chloroform, from one batch) was added and the chloroform evaporated under nitrogen (by rotating the vial) to obtain a thin layer of DLPC film. Tris-HCl buffer (0.6 mL), pH 7.0 was added and the mixture was vortexed for 1 min, resulting in a multilamellar dispersion. The suspension was then transferred to a new screw-capped 1.5 mL HPLC vial with a septum on top and diluted with 800 µL of pre-warmed (50°C) Tris-HCl buffer, pH 7.0. The lipid peroxidation reaction was initiated by adding 200 µL of AAPH **47** (0.2 mM in water) solution to a final volume of 1.5 mL to minimize head space oxidation. The vial was sampled through the septum via syringe to minimize the introduction of air to the system. Oxidations were carried out at 50°C in the dark; a control reaction was performed in the absence of BHT **17**.

Conjugated diene formation was measured by removing 30 µL aliquots, which were dissolved in 970 µL methanol to a final volume of 1.0 mL. These aliquots were removed at 2 min intervals for 20 min and 5 min intervals until 30 min. Absorbance was measured at 240 nm using methanol as a blank. SECO **2** and SDG **1** were analyzed at two different concentrations (0.53 and 0.80 mM). Due to incomplete dissolution of SECO **2** and SDG **1** in hexane, the procedure was

modified slightly. In a vial, 5 mg DLPC in chloroform was added and the chloroform evaporated under nitrogen. To this vial was added a solution of SDG **1** or SECO **2** dissolved in Tris-HCl buffer (0.6 mL). Other oxidation reaction parameters were the same to those for BHT **17**. For each analysis, three individual samples were prepared in duplicate and analyzed using EXCEL program. Errors were expressed as standard deviation of the mean.

3.2.2. Analytical HPLC

The BHT **17**:AAPH **47** oxidation product formation was measured using an Alliance HPLC (Waters Inc., Milford, MA) on a symmetry C18 reverse phase column (3x150 mm, 5mm, Waters Inc.). Mobile phase consisted of 0.05% TFA in H_2O (solvent A) and 0.05% TFA in ACN (solvent B). A gradient elution was performed (Table 3.1) with a flow rate was 0.4 mL/min. Peaks were detected using a 996 UV-Vis PDA detector (Waters Inc.) with full spectral scans (200-400 nm).

Table 3.1. HPLC gradient for stoichiometry ratio.

Time (min)	%H_2O	%ACN
1-2	98	2
3-5	60	40
6-10	98	2

3.3. Experimental 2: Rancidity test

3.3.1. Induction time (h) measurement

Rancidity test was measured using a modification of the method of Martinez-Tome *et al.,* 2001.[279] A pilot study compared SECO **2** to BHT **17** by weight (mg) per 100 g oil (10 mg/100 g oil = 100 mg/kg). For follow-up study, SECO **2**, SDG **1** and BHT **17** were treated with three different concentrations (0.04, 0.06 and 0.08 mM) of antioxidant or control (absence of antioxidant); SDG polymer **3** was treated by weight (10, 20, 30 mg/100 g oil). Samples were stored at room temperature (23°C) in sealed glass containers in the dark to prevent oxidation and were analyzed at 30 day intervals over 120 days using a Rancimat Analyzer (model 743, Metrohm Ltd. Herisau Switzerland). Data are the mean of at least two replications, using EXCEL program for statistical analysis. The Rancimat method,[280,281] has been developed as an automated version of the active oxygen method (AOM)[282] for the determination of the induction time of fats and oils. In this method the oil is heated and highly volatile organic acids (mainly formic acid) are produced by autoxidation of the oil.[280] These are transferred by the stream of air into a measuring vessel containing deionised water, whose conductivity is continually being measured. The time required for a change in conductivity (Delta Kappa uS/cm) is called the induction time (IT).[280] Plotting conductivity against time produces oxidation curves, whose point of inflection is known as the induction time (h), which is automatically recorded by the Rancimat.[280] The inflection point is the point at which all of the antioxidant is consumed and unimpeded lipid peroxidation occurs. IT provides an

estimate of the oxidative stability of the oil being measured. For each analysis, 3 g of each sample in duplicate were removed, added to measuring vessel and subjected to oxidation (110 ± 2°C) by the air stream (flow of 20 L/h).

3.4. Experimental 3: Determination of oxidation compounds of SECO 2 and SDG 1

3.4.1. SECO 2 and SDG 1 oxidation by AAPH 47

SECO **2** or SDG **1** oxidation was performed based on modifications of literature method.[278] Briefly, SECO **2** or SDG **1** (0.01 mol L^{-1}) dissolved in MeOH/H_2O (50/50%, v/v) was incubated with AAPH (0.1 mol L^{-1}) also dissolved in MeOH/H_2O (50/50%, v/v), in 10 mL screw-capped test tubes with septum at 60°C. Aliquots (20 µL) were removed through the septum via syringe (to minimize the introduction of air to the system) at 5.0 min intervals for 1h and 30.0 min intervals to the end of the reaction. The oxidation reaction was monitored using analytical HPLC for 6 h to optimize the reaction time, 5 h for SECO **2** and 4 h for SDG **1** was enough to form sufficient levels of oxidation products. Each aliquot was analyzed by HPLC as described in section 3.4.2. The reactions with SECO **2** and SDG **1** were scaled up in order to collect enough quantity of each oxidation product for further purification and structural determination, using semi-prep HPLC and prep-HPLC followed by LC-MS and NMR. SECO **2** (0.01 mol L^{-1}) was dissolved in MeOH/H_2O (50/50%, v/v) and incubated with AAPH **47** (0.1 mol L^{-1}) in a solution of MeOH/H_2O (50/50%, v/v), under atmospheric oxygen, using a 1000 mL glass

bottle. This mixture was heated in the dark at 60°C for 5h, using a shaking water bath. SDG **1** (0.01 mol L^{-1}) was dissolved in MeOH/H_2O (50/50%, v/v) and incubated with AAPH **47** (0.1 mol L^{-1}) in a solution of MeOH/H_2O (50/50%, v/v), under atmospheric oxygen, using a 1000 mL glass bottle. This mixture was heated in the dark at 60°C for 4h, using a shaking water bath. AAPH **47** in the absence of SECO **2** or SDG **1** was used as control.

3.4.2. Isolation and purification

3.4.2.1. Analytical HPLC

Oxidation products of SECO **2**/SDG **1** were separated using an Alliance HPLC (Waters Inc., Milford, MA) on a symmetry C18 reverse phase (RP) column (3x150 mm, 5mm, Waters Inc.). Mobile phase consisted of 0.05% trifluoroacetic acid (TFA) in H_2O (solvent A) and 0.05% TFA in acetonitrile (ACN) (solvent B). A gradient elution was performed (Table 3.2) with a flow rate at 0.4 mL/min. Peaks were detected using a 996 UV-Vis photodiode array (PDA) detector (Waters Inc.) with full spectral scans (200-400 nm).

Table 3.2. Analytical HPLC gradient for SECO **2** (a) and SDG **1** (b) oxidation products.

(a)

Time (min)	%H_2O	%ACN
1-10	90	10
30	60	40
40	40	60
50-55	90	10

(b)

Time (min)	%H_2O	%ACN
1-5	90	10
20	60	40
30	90	10
35	90	10

3.4.2.2. Preparative HPLC

Separations were performed on a C18 (Prep Nova-Pak®, Waters Inc.), 6µm, 25x10 cm RP column with a flow rate of 20 mL/min; other parameters were similar to analytical HPLC with the exception of using methanol instead of ACN. In prep-HPLC, 23 fractions of SECO **2** oxidation products were collected; fractions 2-6 and 17-22 were enriched with the compounds of interest. Since these fractions were still not pure, they were combined and subsequently purified using semi-prep HPLC. To isolate SDG **1** oxidation products only semi-prep HPLC was used.

3.4.2.3. Semipreparative HPLC

Separations were performed on a C18, 5µm, 300x10 mm RP column with a flow rate of 3.0 mL/min. A gradient method, which was used for SECO **2** and SDG **1** oxidation products, is shown in (Table 3.3-3.4); other parameters were similar to the analytical HPLC protocol.

Table 3.3. Semi-prep HPLC gradient for SECO **2** oxidation products.

Time (min)	%H_2O	%ACN
1-10	80	20
50-55	60	40
56-60	20	80
61-65	80	20

For SECO **2**, 120 fractions were collected using a fraction collector. The fractions containing compounds of interest are listed:

Compound **48** (RT = 25.6), obtained from preparative fractions 61-65;

Compound **49** (RT = 26.7), obtained from preparative fractions 66-69;

Compound **50** (RT = 18.6), obtained from preparative fractions 24-30;

Compound **51** (RT = 19.6), obtained from preparative fractions 35-37;

Compound **52** (RT = 28.9), obtained from preparative fraction 22;

Compound **53** (RT = 10.7) and **54** (RT = 11.4), obtained from preparative fractions 2-6.

Table 3.4. Semi-prep HPLC gradient for SDG **1** oxidation products.

Time (min)	%H_2O	%ACN
1-10	95	5
60-65	60	40
66-72	5	95
73-75	95	5

For SDG **1**, 120 fractions were collected using a fraction collector. The fractions containing compounds of interest are listed:

Compound **55** (RT = 14.3), obtained from preparative fractions 72-73;

Compound **56** (RT = 12.9), obtained from preparative fractions 64-65;

Compound **57** (RT = 12.1), obtained from preparative fractions 62-63;

Compound **58** (RT = 17.0), obtained from preparative fractions 84-92

3.5. Identification of oxidation compounds

3.5.1. LC-MS analysis

LC-MS were determined using a Quattro-LC (Micromass UK Limited, Manchester, UK) equipped with ES-MS (±) sources (Micromass) using ACN as mobile phase. LC analysis was performed applying the same gradient as analytical HPLC except 0.05% formic acid was used instead of 0.05% TFA.

3.5.2. NMR analysis

The NMR data was obtained on a Bruker AVANCE DPX-500 spectrometer (PBI in Saskatoon, SK) operating at 500 MHz and 125 MHz for proton and carbon, respectively. Deuterated methanol (CD_3OD) was used as solvent. Residual signals from CD_3OD (3.30 ppm and 49.0 ppm for proton and carbon-13) served as internal standard. Programs for 2-D experiments were available from a software package (XWINNMR) provided by Bruker. COSY, HMQC and HMBC experiments were performed with gradient pulses. The Distortionless Enhancement by Polarization Transfer (DEPT) experiment together with 2-dimentional-NMR (2D-NMR) experiments including: Correlation Spectroscopy (COSY), Heteronuclear Multiple Quantum Correlation (HMQC), Heteronuclear Multiple Bond Correlation (HMBC) and Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were performed with gradient pulses.

3.6. Extinction coefficient

Extinction coefficients of samples (in methanol) were obtained from the following equation:

$$\varepsilon = A/CL \qquad \text{(equation 27)}$$

Where ε is extinction coefficient (L $mole^{-1}$ cm^{-1}), A is maximum absorption (at 280 nm), C is concentration (mole), L is path length of cell which was 1cm. For each analysis five individual samples with five replications were used using EXCEL (Microsoft Office, Office 10) program for statistical analysis.

3.7. Peroxide Value test

An ammonium thiocyanate assay was used based on a modification of the method of Asamari, 1996.[283] The assay was conducted by adding 2.5 mL of 75% ethanol, 0.05 mL of ammonium thiocyanate solution (30%w/v in H_2O), and 0.05 mL of ferrous chloride (0.1%w/v in H_2O) to 100 μL of sample in ethanol. In a 3 mL test tube, the mixed solution was incubated at room temperature (23°C) in the dark for 3 min. The absorbance of the reaction mixture was measured at 500 nm against 75% ethanol as a blank. Peroxide concentrations were determined according to the equation obtained from the standard curve of H_2O_2. To obtain the standard curve, H_2O_2 at concentrations ranging from 5-40 μg/mL were added to the reaction mixture in a reference cell. In the reference cell, previously added 2.5 mL 75% ethanol, 0.05 mL of ammonium thiocyanate solution (30%w/v in H_2O), and 0.05 mL of ferrous chloride to a final volume of 3 mL. Peroxides were calculated from the ratio of

concentration of each sample to reference. Results are an average of four individual readings (from four separate tubes) at 500 nm, using UV/VIS spectrophotometer (Agilent 8453, Canada) with Agilent Chem Station software. EXCEL program was used for statistical analysis.

4.0. RESULTS

4.1. Antioxidant capacity of flaxseed lignans in liposomal and vegetable oil systems

4.1.1. Stoichiometry ratio determination

The stoichiometric ratio (*n*) for peroxyl radical trapping is calculated from equation 24 (section 3.2.1 for details). BHT **17** (n = 2) was used as a standard for antioxidant activity to determine an *Ri* value for AAPH **47**-mediated lipid peroxidation of DLPC liposomes at 50°C (Table 4.1).[278] An *n* of 2.0 ± 0.01 was confirmed for BHT **17** by analyzing products of the BHT **17**/AAPH **47**/DLPC reaction. The *Ri* value was used to calculate *n* for each antioxidant (Table 4.1-4.2). In addition, under these conditions n = 1.75 was determined for α-tocopherol (personal communication, Brian Fahlman). In the presence of antioxidant, lag time was determined from the intercept of two lines representing the delay prior to the onset of lipid peroxidation and the oxidation phase, when the antioxidant had been consumed (BHT **17**, SECO **2** or SDG **1**). This is demonstrated in Figure 4.20, where the line representing delay (y = 0.002X + 0.2108) and the line representing the onset of lipid peroxidation (y = 0.012X + 0.0041) in the presence of 0.8 mM BHT **17** are shown.

Plots of absorption (240 nm) vs time (min) for oxidations of Blank, BHT **17**, SECO **2** and SDG **1** in liposomes are depicted in Figure 4.1-4.2 and described as follows:

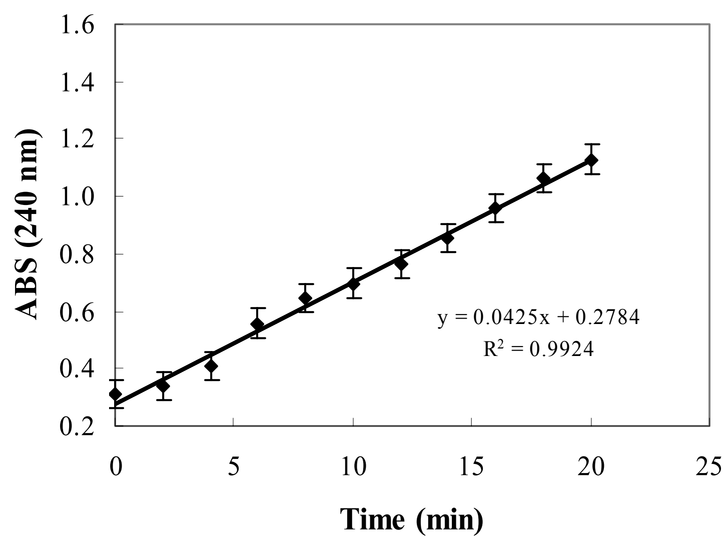


Figure 4.1. Mean (±SD) graphs of determination of antioxidant-mediated lag time (min)AAPH **47**-mediated peroxidation of DLPC liposomes (pH 7.4, 50°C), n=3.

Blank: There was no delay time (induction time) for blank since it did not contain any antioxidant (Figure 4.1). Therefore, a plot of absorption (240 nm) vs time (min) for a control was linear for conjugated diene formation over 20 min.

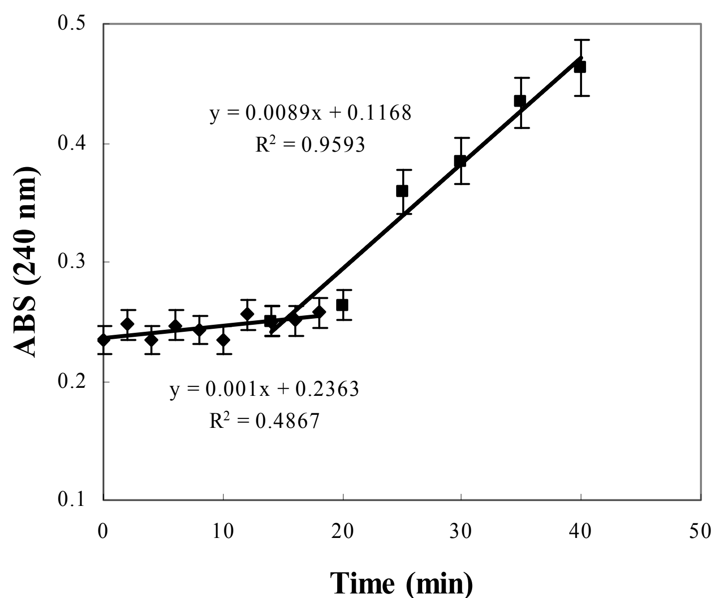
BHT 17: delayed AAPH **47**-induced lipid autoxidation by 15.3 min (0.53 mM) and 17.2 min (0.80 mM) (Figure 4.2).

SECO 2: delayed AAPH **47**-induced lipid autoxidation by 11.7 min (0.53 mM) and 13.4 min (0.80 mM) (Figure 4.3)

SDS 1: delayed AAPH **47**-induced lipid autoxidation by 8.5 min (0.53 mM) and 10.5 min (0.80 mM) (Figure 4.4).

The stoichiometric ratios for SECO **2** was 1.5± 0.15 and for SDG **1** was 1.1-1.2 ± 0.09 (Table 4.2). These results indicate that both SECO **2** and SDG **1** are less potent antioxidants than BHT **17**, although SECO **2** is superior to SDG **1**.

(0.53 mM BHT **17**)



(0.80 mM BHT **17**)

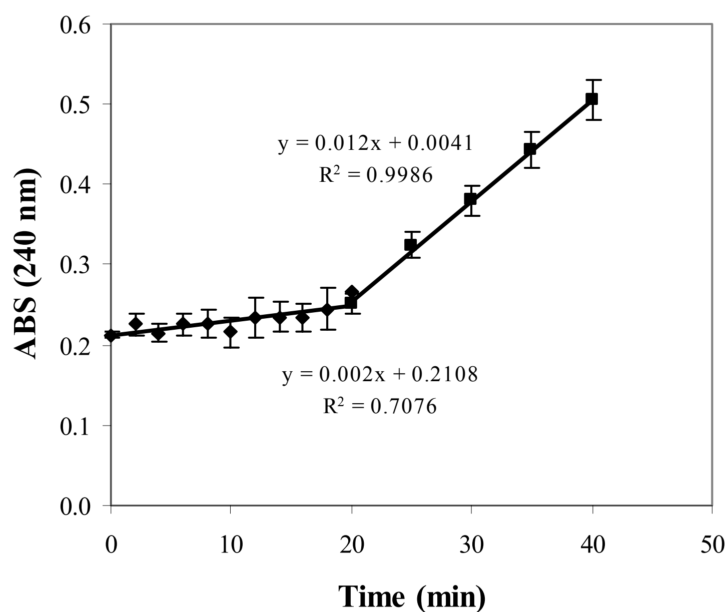
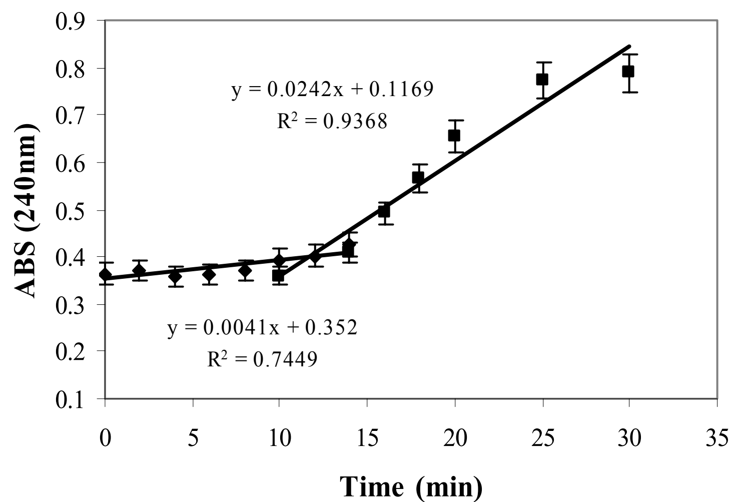


Figure 4.2. Mean (±SD) graphs of determination of antioxidant-mediated lag time (min) at pH 7.4, 50°C, at 0.53 and 0.8 mM for BHT **17** (n=3).

(0.53 mM SECO **2**)



(0.80 mM SECO **2**)

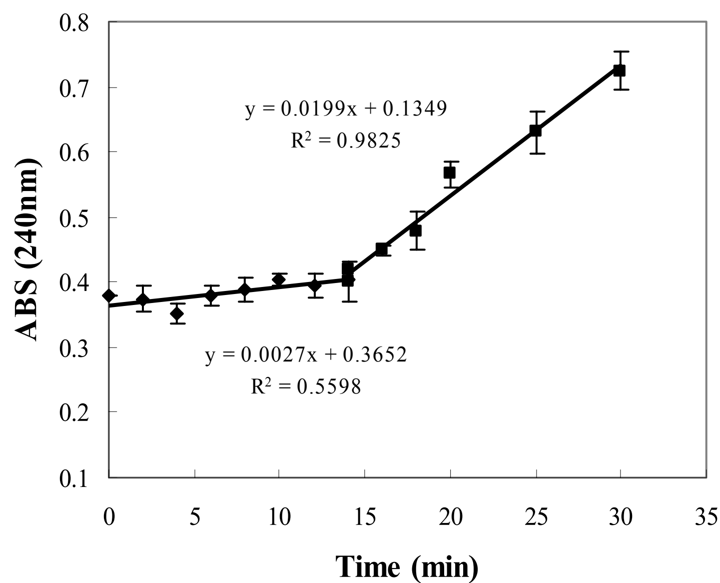
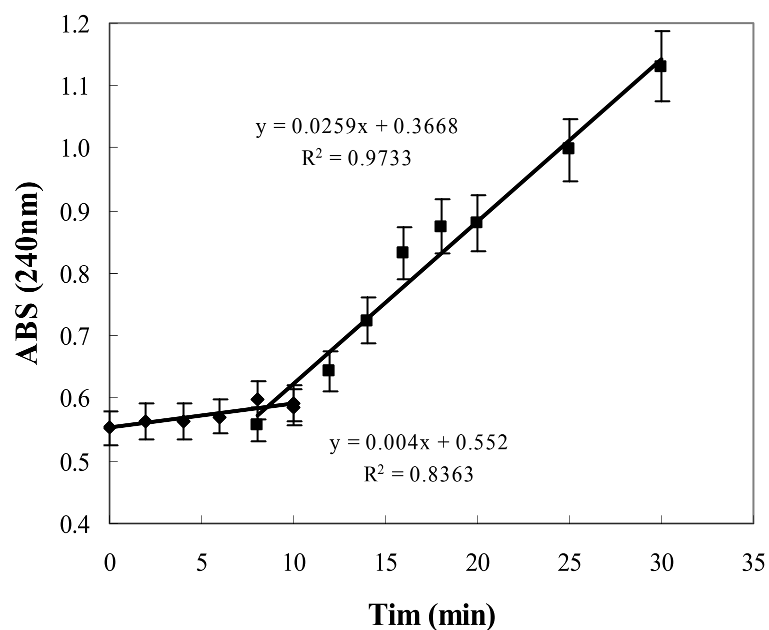


Figure 4.3. Mean (±SD) graphs of determination of antioxidant-mediated lag time (min) at pH 7.4, 50°C, at 0.53 and 0.8 mM for SECO **2** (n=3).

(0.53 mM SDG **1**)



(0.80 mM SDG **1**)

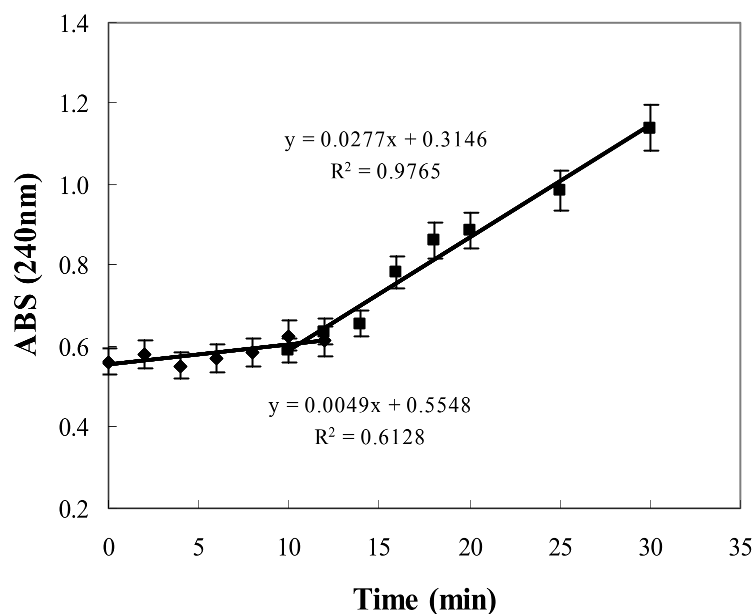


Figure 4.4. Mean (±SD) graphs of determination of antioxidant-mediated lag time (min) at pH 7.4, 50°C, at 0.53 and 0.8 mM for SDG **1** (n=3).

Table 4.1. Mean (±SD) rate of AAPH **47**-induced lipid peroxidation of DLPC at pH 7.4, 50°C calculated in the presence of BHT **17** (n = 3).

sample at 50°C	Lag time (min)	*Ri* value (mM min^{-1})	*n*
BHT 17 (0.53 mM)	15.3 ± 0.01	0.07 ± 0.01	2.0 ± 0.01
BHT 17 (0.80 mM)	17.2 ± 0.04	0.09 ± 0.01	2.0 ± 0.01

Table 4.2. Mean (±SD) stoichiometric ratio (*n*) for SECO **2** and SDG **1** at 50°C (n=3).

sample at 50°C	Lag time (min)	*Ri* value (mM min^{-1})	*n*
SECO 2 (0.53 mM)	11.7 ± 0.10	0.07 ± 0.01	1.5 ± 0.10
SECO 2 (0.80 mM)	13.4 ± 0.10	0.09 ± 0.01	1.5 ± 0.20
SDG 1 (0.53 mM)	8.5 ± 0.03	0.07 ± 0.01	1.1 ± 0.15
SDG 1 (0.80 mM)	10.5 ± 0.05	0.09 ± 0.01	1.2 ± 0.03

4.1.2. Antioxidant activity of flaxseed lignans in vegetable oil

Antioxidant activity of flaxseed lignans was accomplished by measuring the induction time for autoxidation of canola oil in the absence or presence of an antioxidant. A pilot study compared SECO **2** to BHT **17** by weight (mg) per 100 g oil (10 mg/100 g oil = 100 mg/kg) (Table 4.3a-4.3b) at two different temperatures (110ºC and 120ºC). Induction times at time = 0.0 was the same for control, BHT **17** and SECO **2** at all amounts. Induction time decreased vs storage time for control, whereas longer induction times were observed for all amounts of both BHT **17** and SECO **2** treated oils. At both temperatures, protection against autoxidation was weight dependent as induction times changed the most over time for 10 mg of BHT **17** and SECO **2** and both produced similar induction times at all amounts.

A follow-up study was carried out which compared the ability of BHT **17** and flaxseed lignans to protect against autoxidation in our model system on a per mole basis. The influence of BHT **17**, SECO **2** and SDG **1** on induction time was measured using 0.04, 0.06 and 0.08 mmole/ 100 g oil. In addition, SDG **1** polymer, whose structure consists of SDG **1** units liked via HMGA moieties, was included in this study.[30]

The results shown in Table 4.4 indicate that induction time increased over time for control and all antioxidants. In addition, each antioxidant produced amount-dependent increase in induction time as compared to control. At all amounts, SECO **2** was comparable to BHT **17** in its ability to maintain induction time at all time points.

Table 4.3a. Induction time (hours) vs storage time (days) at 110ºC for 10, 20 and 30 mg antioxidant/100 g oil.

			Induction time (h)				
Storage Time (days)	**Control**	**SECO 2 (10 mg)**	**SECO 2 (20 mg)**	**SECO 2 (30 mg)**	**BHT 17 (10 mg)**	**BHT 17 (20 mg)**	**BHT 17 (30 mg)**
0	11.29	11.26	11.28	11.25	11.33	11.29	11.36
30	10.38	10.68	10.98	11.13	10.61	11.05	11.17
60	9.93	10.19	10.44	10.98	10.22	10.86	11.11
90	8.62	9.77	10.21	10.62	9.92	10.50	10.82
120	7.09	9.01	9.16	10.03	9.05	9.44	10.27

Table 4.3b. Induction time (hours) vs storage time (days) at 120ºC for 10, 20 and 30 mg antioxidant/100 g oil.

	Induction time (h)						
Storage Time (days)	**Control**	**SECO 2 (10 mg)**	**SECO 2 (20 mg)**	**SECO 2 (30 mg)**	**BHT 17 (10 mg)**	**BHT 17 (20 mg)**	**BHT 17 (30 mg)**
0	5.66	5.65	5.66	5.66	5.66	5.65	5.67
30	4.91	5.07	5.18	5.30	5.18	5.31	5.35
60	4.58	4.70	4.87	5.17	4.98	5.06	5.21
90	4.02	4.63	4.68	4.97	4.67	4.78	5.09
120	2.83	3.98	4.15	4.32	4.20	4.44	4.66

Table 4.4. Induction time (hours) vs storage time (days) at 110ºC of BHT **17**, SECO **2** and SDG **1** at 0.04, 0.06 and 0.08 mmol/100 g oil. SDG polymer **3** was 10, 20, 30 mg/100 g oil.

Storage Time (days)	**Control**	**SECO 2 (0.04mM)**	**SECO 2 (0.06mM)**	**SECO 2 (0.08mM)**	**BHT 17 (0.04mM)**	**BHT 17 (0.06mM)**	**BHT 17 (0.08mM)**
0	11.43	11.45	11.60	11.61	11.45	11.60	11.63
30	10.06	11.12	11.46	11.27	11.22	11.40	11.59
60	8.90	10.52	10.93	11.16	10.72	11.16	11.51
90	7.74	9.93	10.42	10.65	10.14	10.68	11.07
120	6.76	9.37	9.95	10.23	9.46	10.39	10.65
Storage Time (days)	**Control**	**SDG 1 (0.04mM)**	**SDG 1 (0.06mM)**	**SDG 1 (0.08mM)**	**SDG polymer 3 (10 mg)**	**SDG polymer 3 (20 mg)**	**SDG polymer 3 (30 mg)**
0	11.43	10.96	11.25	11.35	11.41	11.50	11.50
30	10.06	10.63	10.84	11.10	10.82	11.02	11.24
60	8.90	10.00	10.70	10.78	10.31	10.60	10.91
90	7.74	9.36	10.36	10.17	9.70	10.19	10.58
120	6.76	8.86	9.25	9.80	9.10	9.70	10.18

Most notably at 120 days the lowest amount used (0.04 mmol/100g oil) of BHT **17** and SECO **2** display almost identical induction times (9.46 h, 9.37 h respectively). In contrast, at all amounts and time points, SDG **1** did not maintain induction time to the same extent as SECO **2** and BHT **17** (Table 4.4). SDG **1** polymer however, produced amount-dependent increases in induction time as compared to control, which were greater than that afforded by SDG **1** and comparable to that seen with SECO **2** (Table 4.4).

4.1.3. Consistency between two model systems

Both model systems showed that as an antioxidant, SECO **2** provides better protection than SDG **1**. The IT ratio from the stoichiometric ratio (calculated from Table 4.1-4.2) were close with the IT ratio from Rancimat experiments (calculated from Table 4.4, values at 120 days). Calculated IT ratios between two model systems are shown in Table 4.5.

Table 4.5. Calculated stoichiometric ratio (*n-value*) and calculated protection factor (PF) value from rancimat.

Antioxidant	*n-value* Stoichiometric ratio	PF Rancimat
SECO	1.5	1.5
SDG	1.2	1.3
SDG polymer	nd	1.4
BHT	2.0	1.6

4.2. Time-course of an AAPH 47-mediated oxidation of SECO 2

Oxidation over time of SECO **2** (0.01 mol L^{-1}) with AAPH **47** (0.1 mol L^{-1}) produced five major dominant UV-detectable peaks (200-400 nm) (Table 4.6). As SECO **2** was oxidized by AAPH **47**, two compounds **50** and **51,** more polar than SECO **2**, and three compounds **48**, **49** and **52,** less polar than SECO **2**, were generated (Table 4.6).

Time course of the oxidation indicated a time dependent formation of oxidation products where compounds **48** and **52** are formed early in the reaction and decomposed after 90 minutes (Figure 4.6a). Meanwhile, compounds **49, 50,** and **51** were formed later (Figure 4.6a). Compound **52** formed at the initial stage of the oxidation reaction, then declined after 120 min. Compound **51** was formed after 90 min and gradually increased to the end of the reaction. Among all of the oxidation compounds, compound **50** was the major compound of the oxidation reaction, which formed after 30 min, increased to a maximum level up to 240 min, then started to decrease after this time (Figure 4.6b). Compound **48** formed early, increased to a maximum level at 90 min and then decreased after this time. Compound **49** was detected after 120 min and gradually increased until the end of the reaction. Control reactions were performed in the absence of AAPH **47** or SECO **2**.

Table 4.6. Oxidation compounds of SECO **2** (0.01 mol L^{-1}) in the presence of AAPH **47** (0.1mol L^{-1}) for 5 h at 60°C.

Compound	**Trivial name**	**RT[a] (min)**	**MW[b]**	**UV absorbance maxima (nm)**
Compound **2**	SECO	21.4	362.4	205.7, 279.8, 224.7 [sh]
Compound **48**	9,9″-SECO dimer	25.6	722.6	205.7, 279.8, 223.8 [sh]
Compound **49**	5,5″-SECO dimer	26.7	722.6	205.7, 279.8, 224.7 [sh]
Compound **50**	4-AP-SECO	18.6	446.6	202.2, 258.5, 223.3 [sh]
Compound **51**	4-AP, 9,9″-peroxide-SECO	19.6	478.6	202.2, 265.6, 223.3 [sh]
Compound **52**	9,9″-peroxide-SECO	28.9	394.2	202.2, 279.8, 226.6 [sh]
Compound **53**	methylene AAPH	10.7	_	198.7, 294.0
Compound **54**	methyl AAPH	11.4	_	202, 294.0

[a]RT, retention time
[b]MW, molecular weight, calculated from mass spectral data
[sh] shoulder

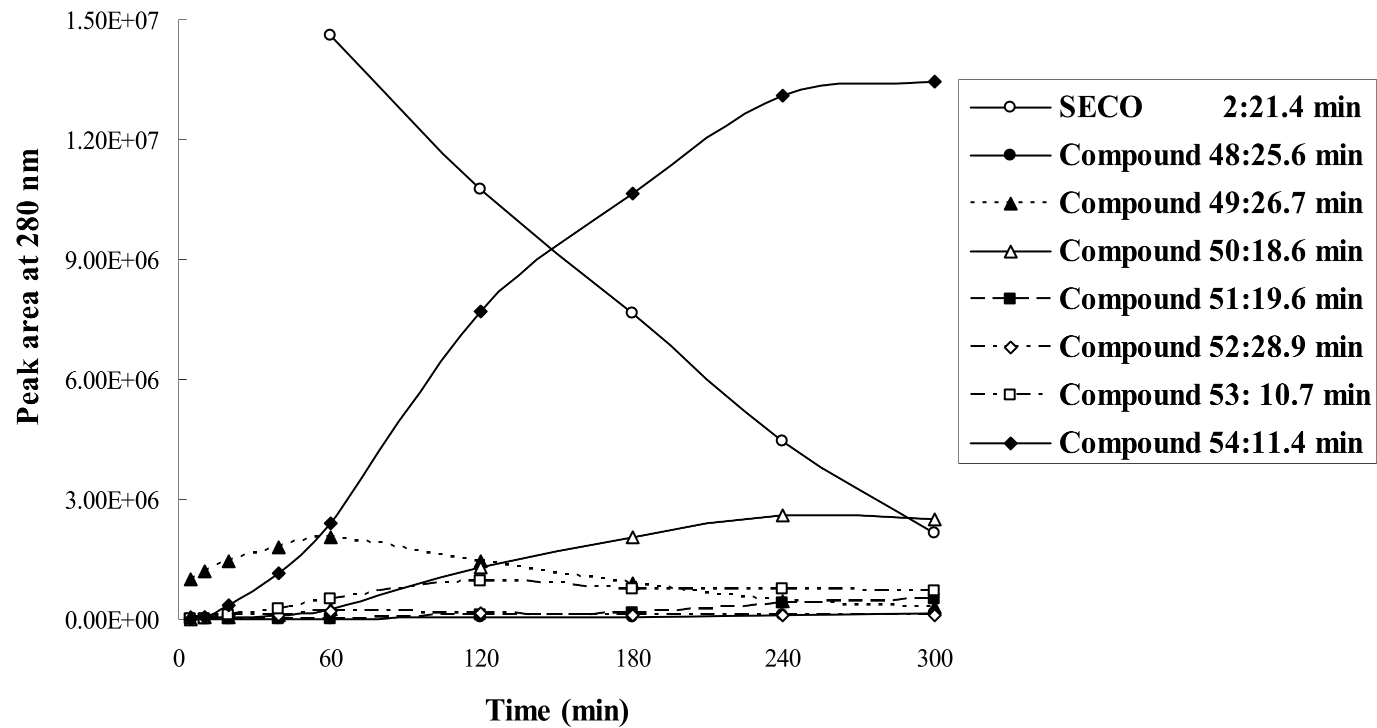


Figure 4.6a*. Oxidation of SECO **2** (0.01 mol L^{-1}) by AAPH **47** (0.1mol L^{-1}) for 5 h at 60°C.
*4.6a shows non-expanded peak areas of oxidation compounds.

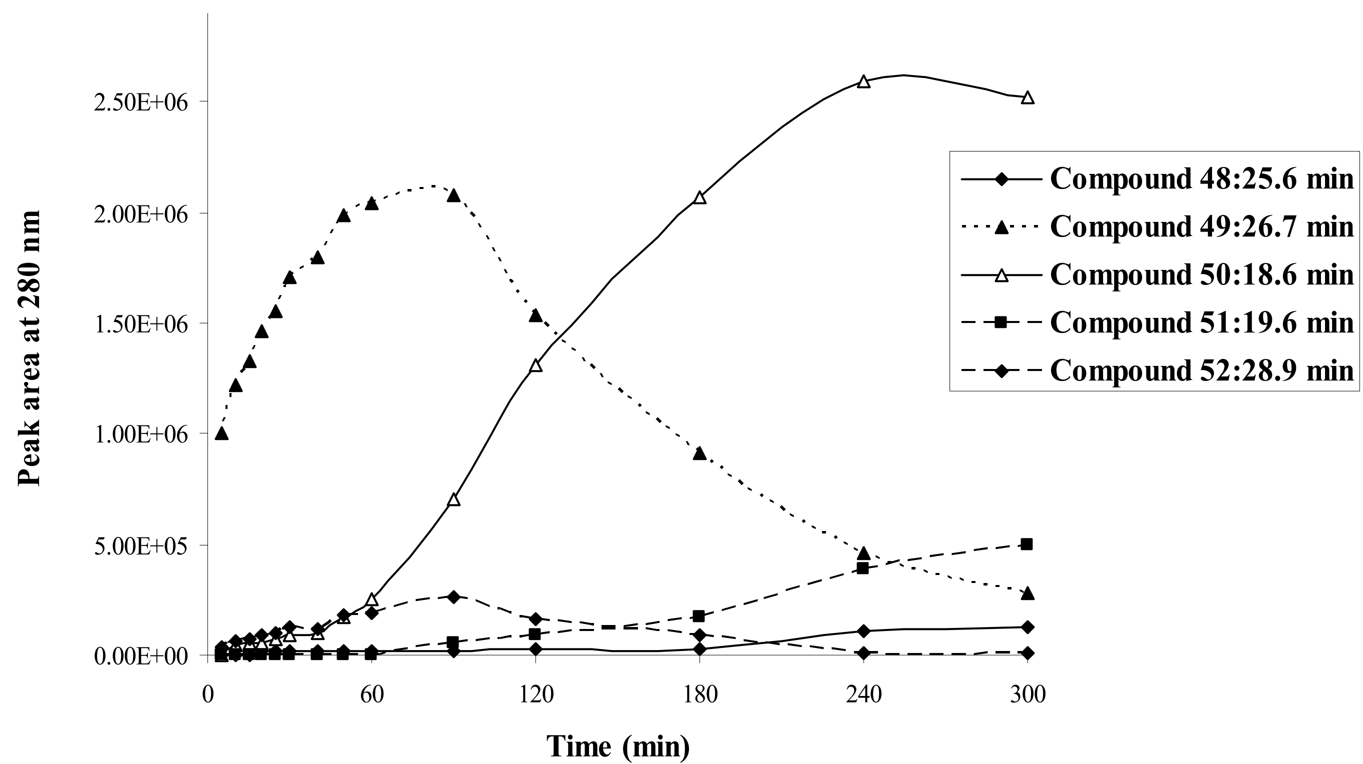


Figure 4.6b*. Oxidation of SECO **2** (0.01 mol L^{-1}) by AAPH **47** (0.1mol L^{-1}) for 5 h at 60°C.
*For simplicity, 4.6b shows expanded peak areas of oxidation compounds.

In the absence of AAPH **47**, no oxidation products were observed from SECO **2**. In the absence of SECO **2**, AAPH **47** was converted to two major oxidation compounds, **53** and **54** (Figure 4.6a).

4.3. Product yield of an AAPH 47-mediated oxidation of SECO 2

SECO **2** (0.01 mol L^{-1}) was oxidized by AAPH **47** (0.1mol L^{-1}) to produce five major oxidation compounds **48** to **54** for a total of 1217 mg (Table 4.7). The oxidation reaction formed some minor compounds (Table 4.7). 270 mg of SECO **2** were recovered after the reaction was terminated at 5 hours. Overall, 138 mg of SECO **2**-containing oxidation reaction materials were lost during purification. The UV-detectable profile by HPLC for oxidation of SECO **2** by AAPH **47** is illustrated in Figure 4.7.

4.4. Structural determination

4.4.1. SECO 2

The results from the ^{1}H- and ^{13}C-NMR for SECO **2** are shown in Table 4.8 and are similar to those obtained from previous studies.[24,284,285] The ^{1}H NMR spectrum exhibited an ABC (three spin-coupled systems) for the aromatic protons and the methoxy singlet at δ 3.66 (Table 4.8). The presence of a total number of 10 hydrogen atoms indicated that SECO **2** has an axis of symmetry. The mass spectra confirmed a pseudo-molecular ion peak at *m/z* 361.4 $[M\text{-}H]^{-}$ and *m/z* 363.4 $[M\text{-}H]^{+}$.

Table 4.7. Yield of oxidation compounds from SECO **2** (0.01 mol L^{-1}) by AAPH **47** (0.1mol L^{-1}) for 5 h at 60°C.

Oxidation compound	**Yield (mg)**	**Yield (%)**
Major compounds		
Compound **48**	225	11
Compound **49**	310	15
Compound **50**	224	10
Compound **51**	112	5
Compound **52**	175	8
Total major compounds	1217	49
Total minor compounds	875	35
Unoxidized SECO	270	10.5
Overall loss	138	5.5

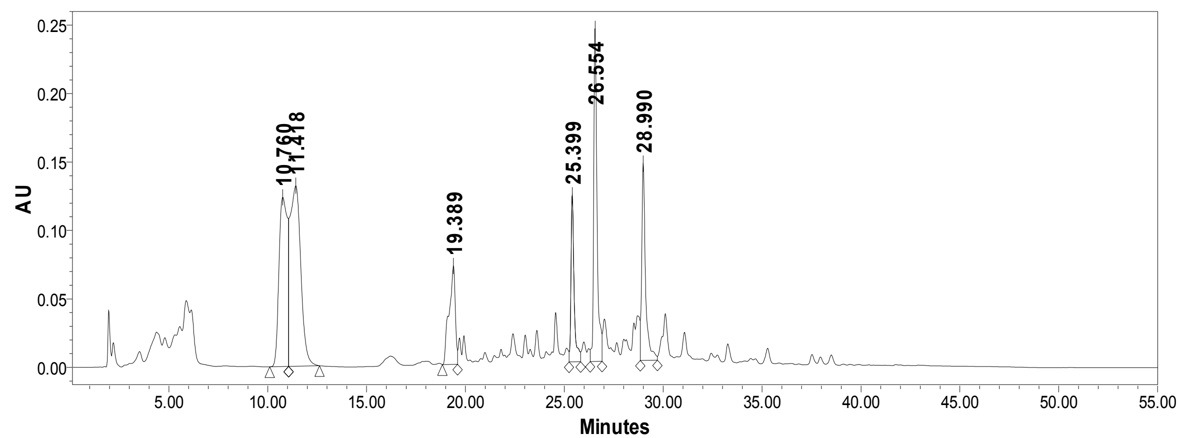


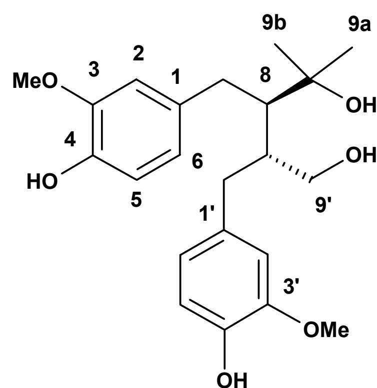
Figure 4.7. HPLC chromatogram from oxidation of SECO **2** (0.01 mol L^{-1}) by AAPH **47** (0.1mol L^{-1}) at 60°C after 5 h, λmax at 280 nm.

The DEPT experiment together with 2D-NMR experiment including: COSY, HMQC, HMBC and NOESY experiments confirmed the presence of two methylenes, three methines, one methyl and three quaternary carbons for a total of nine signals (Table 4.8). From the chemical shifts, the quaternary carbons may be attributed to the tri-substituted aromatic ring. The distinction between C-3/3′ and C-4/4′ was achieved from the ^{3}J (C,H) coupling of C-3/3′ with the methoxy protons. In addition, COSY and HMQC spectra verified an ABC spin system, together with an isolated methoxy group that corresponds to the protons of the methylenes and/or methines of C-9, C-8, C-7 and the aromatic system.

4.4.2. SECO 2 oxidation compounds

4.4.2.1. Identification of compound 48 (9, 9”-SECO dimer)

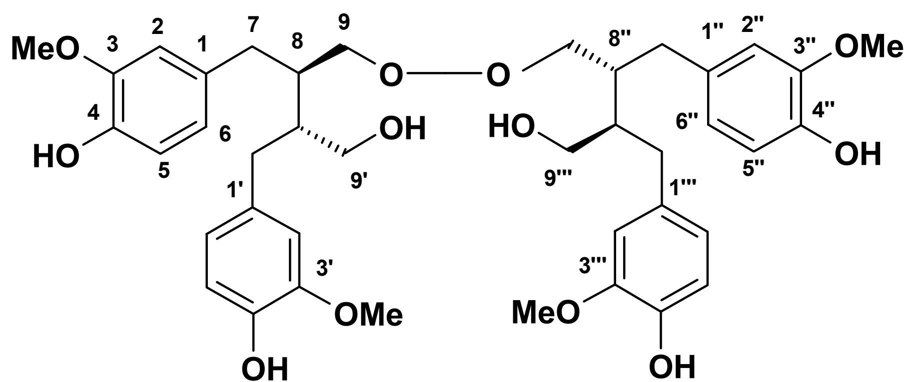
Compound **48** (RT=25.6 min) showed pseudo-molecular ions (*m/z* 721.6 $[M-H]^-$, 723.6 $[M+H]^+$), reflective of a dimerization of SECO **2** (Figure 4.8). The molecular formula of **48** was calculated to be $C_{40}H_{50}O_{12}$. The ^{13}C NMR spectrum displayed signals for 20 carbon atoms suggesting that compound **48** may have an axis of symmetry (Table 4.9). Chemical shifts of the aliphatic moieties and that of one of the aromatic rings showed some similarity to those of the corresponding carbons in SECO **2**. However, ^{13}C NMR indicated that distinguishable differences were observed for C-9 (74.4), C-8 (47.6) and C-7 (39.9) compared to 62.1, 44.1 and 36.0 for the same carbons in SECO **2**. This is reflective of two sub-structures linked via -OH moieties on the aliphatic part of the molecule.



SECO **2**
$C_{20}H_{26}O_6$

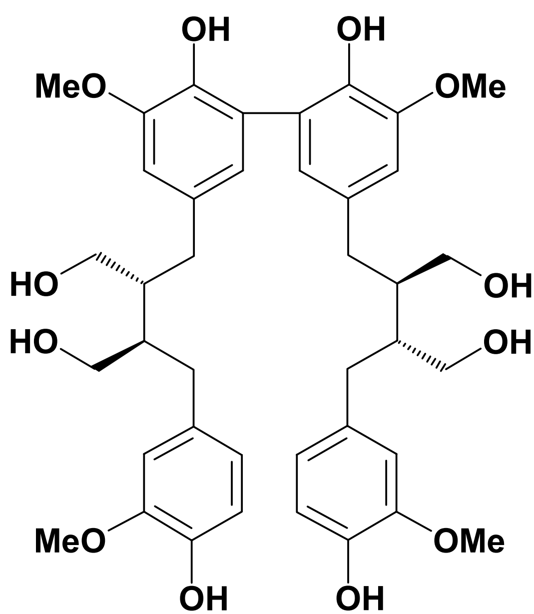
Table 4.8. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of SECO **2** in CD_3OD.

SECO 2			
Position	δ_C	δ_H **(multiplicity, *J* Hz)**	**HMBC**
1/1′	133.9	-	5, 9a/9b, 8/5′, 9′a/9′b, 8′
2/2′	115.8	6.52 (d, 2.0)	6, 7a/7b/6′, 7′a/7′b
3/3′	148.8	-	5, 2, OCH3/5′, 2′, OCH3
4/4′	145.5	-	5, 2, 6/5′, 2′, 6′
5/5′	113.4	6.59 (d, 8.0)	-
6/6′	122.7	6.47 (dd, 8.0)	7a/7b, 2
7a/7′a	36.0	2.48 (dd, 7.8, 13.8)	2, 6, 8, 9a/9b
7b/7′b	36.0	2.59 (dd, 6.9, 13.8)	2′, 6′, 8′, 9′a/9′b
8/8′	44.1	1.83 (br t, 6.1)	9a/9b, 7a/7b/9′a/9′b, 7′a/7′b
9a/9′a	62.1	3.54 (dd, 5.0, 11.0)	8, 7a/7b, 9b/9′b
9b/9′b	62.1	3.49 (dd 4.9, 11.1)	8′, 7′a/7′b, 9a/9′a
OCH_3	56.2	3.66 (s)	-



48

Figure 4.8. Proposed structure for compound **48**.



49

Figure 4.9. Proposed structure for compound **49**.

Table 4.9. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of **48** and **49** in CD_3OD**.**

Compound		**48**		**49**
Position	δ_C	δ_H **(multiplicity, *J* Hz)**	δ_C	δ_H **(multiplicity, *J* Hz)**
1,1″	133.6	-	133.8	-
2,2″	115.5	6.54 (d, 1.5)	115.9	6.60 (d, 1.5)
3,3″	149.3	-	149.3	-
4,4″	145.4	-	146.7	-
5,5″	113.2	6.58 (d, 8.0)	127.0	-
6,6″	122.6	6.48 (d, 1.5)	123.2	6.54 (d, 1.5)
7,7″	39.9	2.62 (m)	36.2	2.60 (m)
8,8″	47.6	2.20 (t, 6.1)	44.0	1.95 (brt, 6.1)
9,9″	74.4	3.65 (m, 4.5, 10.5)	62.1	3.60 (m, 4.5, 10.5)
3, 3″-OCH_3	56.5	3.77 (s)	56.4	3.77 (s)
1′, 1‴	133.9	-	133.4	-
2′,2‴	115.8	6.54 (d, 1.5)	115.8	6.54 (d, 1.5)
3′,3‴	148.8	-	148.8	-
4′,4‴	145.4	-	145.4	-
5′,5‴	113.1	6.58 (d, 8.0)	113.3	6.59 (d, 8.0)
6′,6‴	122.5	6.47 (dd, 1.5, 8.0)	122.7	6.47 (dd, 1.5, 8.0)
7′,7‴	36.0	2.62 (m)	36.0	2.58 (m)
8′,8‴	44.6	1.83 (brt, 6.1)	44.1	1.95 (brt, 6.1)
9′,9‴	62.2	3.61 (m, 4.7, 10.8)	62.1	3.60 (m, 4.5, 10.5)
3′, 3‴-OCH_3	56.4	3.69 (s)	56.2	3.66 (s)

The ^{1}H NMR spectrum of **48** showed an ABC system [6.54 (dd, 1.5, 8.0), 6.58 (d, 1.5), and 6.48 (d, 8.0)] for the aromatic rings (Table 4.9). From the HMBC spectrum, correlation peaks through H-8 (δ 2.20) to C-1, C-7, C-7′, C-8′, C-9, C-9′ established that a dimer was linked between 9-OH to C-9″ (Table 4.9). A downfield shift of the C-8 protons (δ 2.20) adjacent to the peroxyl linkage compared to the C-8 protons (δ 1.95) near the unsubstituted alcohols (SECO H-8: δ 1.83) suggest that the peroxy link may have caused the molecule to twist to avoid congestion. In doing so, the C-8 proton appears to be in a new shielding environment.

4.4.2.2. Identification of compound 49 (5,5"-SECO dimer)

Compound **49** (RT=26.7 min) showed pseudo-molecular ions (*m/z* 721 [M-H]$^{-}$, 723 [M+H]$^{+}$), reflective of another dimer with a molecular formula of $C_{40}H_{50}O_{12}$ (Figure 4.9). The ^{13}C NMR data indicate that compound **49** is a symmetric molecule (Table 4.9). Distinguishable differences in NMR data were observed for C-4 (146.7) and C-5 (127.0) compared to 145.5 and 113.4 for the same carbons in SECO **2**. All other chemical shifts of the aliphatic and aromatic moieties were very similar to those of the corresponding carbons in SECO **2** (Table 4.8). These chemical shifts indicated that these two sub-structures are linked via aromatic rings. The ^{1}H NMR spectrum of **49** has an ABC system [6.54 (dd, 1.5, 8.0), 6.59 (d, 1.5), and 6.47 (d, 8.0)] and an AB [δ 6.60 and 6.54 (each d, 1.5)] system for aromatic rings (Table 4.9).

In the HMBC spectrum, correlation peaks observed between H-6/H-6″ (δ 6.54) to C-1/C-1″, C-4/C-4″, C-5/C-5″ and C-7/C-7″ established a connection from C-5 to C-5″ for dimerization. In addition, COSY and HMQC spectra of compound **49** verified an ABC spin system, together with an isolated methoxy group, that corresponds to the protons of the methylenes of C-9, C-8 and methines of C-7. The two dimers **48** and **49** would predict to have *n-value*=1 due to the loss of one hydrogen form SECO **2** molecule that undergoes dimerization (section 5.6 for details).

4.4.2.3. Identification of compound 50 (4-AP-SECO)

Compound **50** (RT=18.6 min) produced pseudo-molecular ions (445 $[M-H]^-$ and 447 $[M+H]^+$), reflective of the addition of one AP radical to the SECO **2** molecule (Figure 4.10). The molecular formula of **50** was then calculated to be $C_{24}H_{34}O_6N_2$. Unlike SECO **2**, the ^{13}C NMR spectrum showed that **50** did not possess an axis of symmetry by displaying signals for 23 carbon atoms (Table 4.10). Signals from an AP moiety were found at δ 176.6, 80.1, and 28.1. A large methyl singlet was observed at δ 1.56 plus two close, but distinguishable ABC systems for aromatic protons.

In the HMBC spectrum, no cross-correlation was observed between SECO **2** and the AP moieties thereby suggesting that AP was linked at the 4-OH or 9-OH because the AP carbon chemical shift at δ 80.1 (s) is indicative of linkage to oxygen. The changes observed from the chemical shift of C-1 (140.8), C-3 (154.1), C-4

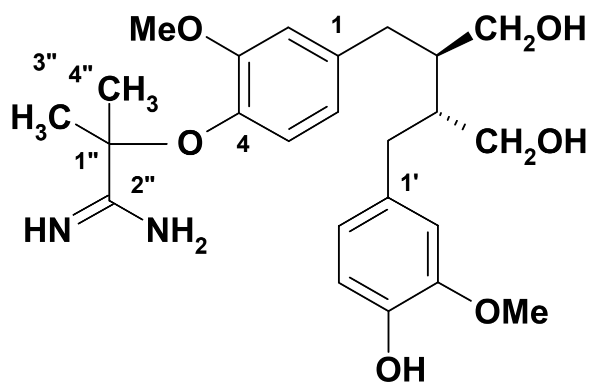
(140.8), C-5 (125.1) and C-6 (124.6) in **50**, compared to 133.9, 148.8, 145.5, and 113.4 for the same carbons in SECO **2**, could be due to the AP moiety on 4-OH (Table 4.10). To confirm this structure, the NOESY spectrum for **50** was obtained. In this spectrum, a cross-correlation peak was observed between the AP methyl groups at δ 1.56 and the aromatic group at δ 6.91 for H-5 (Table 4.10).

4.4.2.4. Identification of compound 51 (4-AP-9,9"-peroxy-SECO)

Compound **51** (RT=19.6 min) produced pseudo-molecular ions (477 $[M-H]^-$ and 479 $[M+H]^+$), reflective of the addition of one AP **47** radical as well as two oxygen atoms to the SECO **2** molecule resulting in a molecular formula of $C_{24}H_{34}N_2O_8$ (Figure 4.11). The carbon chemical shifts of the aliphatic moiety of **51** were similar to those of the same carbons in **52** suggesting the presence of the peroxide function at C-9 and C-9′ (Table 4.10). The ^{13}C NMR spectrum also showed carbon atoms at δ 28.1, 80.1, and 176.6 for an AP residue (Table 4.10). As in **48**, no correlation was observed between the AP and the SECO **2** moieties of **51**. However, the similarity of their aromatic carbon chemical shifts in both ^1H- and ^{13}C-NMR experiments was strong evidence that the AP in **51** was linked to the 4-OH on one of the aromatic rings (Table 4.10). Compounds **50** and **51** would be predicted to have *n*=2 due to first, losing hydrogen (*n*=1) from SECO **2** molecule and second, trapping AP free radical (*n*=1) (section 5.6).

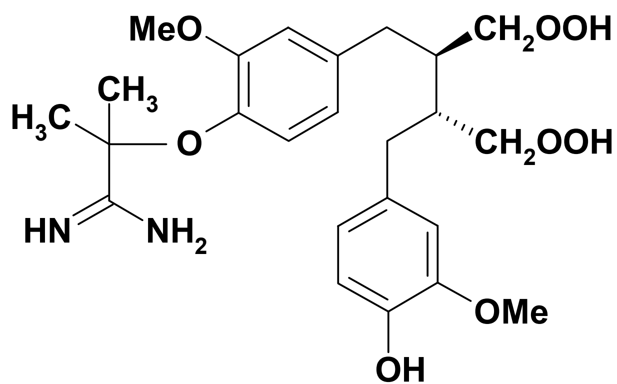
4.4.2.5. Identification of compound 52 (9.9”-peroxy-SECO)

Compound **52** (RT=28.9 min) produced pseudo-molecular ions (393 $[M-H]^-$ and 395 $[M+H]^+$), reflective of the addition of two oxygen atoms to SECO **2** (Figure 4.12). The molecular formula for compound **52** was calculated to be $C_{20}H_{26}O_8$.



50

Figure 4.10. Proposed structure for compound **50**.

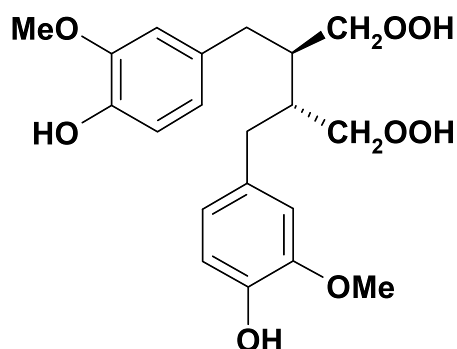


51

Figure 4.11. Proposed structures for compound **51**.

Table 4.10. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of **50** and **51** in CD_3OD.

Compound	**50**		**51**	
Position	δ_C	δ_H **(multiplicity, *J* Hz)**	δ_C	δ_H **(multiplicity, *J* Hz)**
1	140.8	-	140.8	-
2	115.1	6.73 (br s)	114.9	6.71 (br s)
3	154.1	-	154.2	-
4	140.8	-	141.5	-
5	125.1	6.91 (d, 8.0)	125.3	6.92 (d, 8.0)
6	124.6	6.67 (br d, 8.2)	124.5	6.69 (br d, 8.2)
7	36.2	2.68 (m)	40.0	2.74 (m)
8	44.5	1.91 (br s)	47.7	2.18 (m)
9	62.0	3.59 (m)	74.3	3.70 (m)
3-OCH_3	56.2	3.73 (s)	56.2	3.71 (s)
1′	133.9	-	133.8	-
2′	115.9	6.65 (br s)	115.8	6.66 (br s)
3′	148.8	-	148.8	-
4′	145.4	-	145.5	-
5′	113.5	6.64 (d, 7.9)	113.4	6.65 (d, 7.9)
6′	122.6	6.51 (br d, 8.0)	124.7	6.54 (br d, 8.0)
7′	36.0	2.63 (m)	40.1	2.73 (m)
8′	44.0	1.96 (br s)	47.6	2.18 (m)
9′	62.2	3.61 (m, 4.5, 10.5)	74.3	3.75 (m)
3′-OCH_3	56.3	3.69 (s)	56.3	3.70 (s)
1″	80.1	-	80.1	-
2″	176.6	-	176.6	-
3″/4″	28.1	1.56 (s)	28.1	1.56 (s)



52

Figure 4.12. Proposed structure for compound **52**.

Table 4.11. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of **52** in CD_3OD.

Position	**52**		
	δ_C	δ_H **(multiplicity,** ***J*** **Hz)**	**HMBC**
1/1′	133.6	-	5, 9a/9b, 8/5′, 9′a/9′b, 8′
2/2′	115.6	6.64 (d, 2.0)	6, 7a/7b/6′, 7′a/7′b
3/3′	148.8	-	5, 2, OCH3/5′, 2′, OCH3
4/4′	145.5	-	5, 2, 6/5′, 2′, 6′
5/5′	113.4	6.60 (d, 8.0)	-
6/6′	124.8	6.65 (dd, 8.0)	7a/7b, 2
7a/7′a	39.0	2.61 (m)	2, 6, 8, 9a/9b
7b/7′b	39.0	2.61 (m)	2′, 6′, 8′, 9′a/9′b
8/8′	47.7	2.20 (br s)	9a/9b, 7a/7b/9′a/9′b, 7′a/7′b
9a/9′a	74.3	4.42 (dd, 5.0, 11.0)	8, 7a/7b, 9b/9′b
9b/9′b	74.3	4.35 (dd, 4.9, 11.1)	8′, 7′a/7′b, 9a/9′a
OCH_3	56.3	3.65 (s)	-

The ^{13}C NMR data for compound **52** displayed signals for 10 carbon atoms, suggesting that the molecule has maintained its symmetry (Table 4.11). Aromatic chemical shifts of SECO **2** and **52** were quite similar (see Table 4.8 for SECO **2** NMR data). However, major changes were observed between the aliphatic moieties: 39.0 (C-7/C-7′), 47.7 (C-8/C-8′), 74.3 (C-9/C-9′) found for **52** compared to 36.0 (C-7/C-7′), 44.1 (C-8/C-8′), 62.1 (C-9/C-9′) for SECO **2**. These differences indicate that **52** have peroxides at both the C-9 and C-9′ positions. In the ^{1}H NMR and ^{13}C spectra from **52**, distinguishable changes were observed only for the aliphatic moiety (Table 4.11). In addition, COSY and HMQC spectra of compound **52** verified an ABC spin system, together with an isolated methoxy group, that corresponds to the protons of the methylenes of C-9, C-8 and methines of C-7 (Table 4.11). Compound **52** with peroxide in the structure would be estimated to have n=0 because it does not lose any hydrogen or trap any AP free radicals (section 5.6).

4.4.2.6. Identification of compound 53 (methylene-AP) and 54 (methyl AP)

Heating AAPH **47** in the absence of SECO **2** produced two major oxidation compounds, **53** (RT=10.7 min) and **54** (RT=11.4 min) (Table 4.6). The mass of **53** and **54** were less than 100 and that they were not distinguishable from ES-MS. Thus, each structure was characterized by HPLC and NMR analyses (Figure 4.13). The ^{1}H NMR and ^{13}C NMR results for compounds **53** and **54** are summarized in Table 4.12.

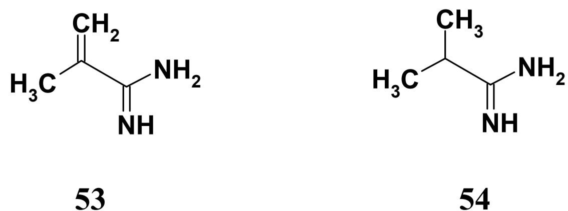


Figure 4.13. Proposed structure for compounds **53** and **54**.

Table 4.12. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of **53** and **54** in CD_3OD.

Position	Compound 53		Compound 54	
	δ_C	δ_H (multiplicity, *J* Hz)	δ_C	δ_H (multiplicity, *J* Hz)
1	18.6	1.90 (s, 3.0)	18.5	1.13 (d, 1.0)
2	120.8	5.36 (s, 1.0)	35.0	2.39 (m, 1.0)
3	139.1	5.72 (s, 1.0)	180.1	-
4	170.8	-	-	-

4.5. Confirmation tests

4.5.1. Oxidation of compound 48

Further oxidation of compound **48** (RT=25.6 min) by AAPH **47** showed the formation of compounds **52**, **51** and SECO **2** from compound **48** (Figure 4.14).

4.5.2. Peroxide value of products 52 and 51

The peroxide value test confirmed the presence of a small quantity of peroxide (3.33 ug mL^{-1}) in compound **52** and (4.6 ug mL^{-1}) in compound **51**, according to the equation obtained from standard curve of H_2O_2 (Figure 4.15). This was equivalent to total of 1.8 ± 0.12 and 1.7 ± 0.09 peroxide equivalents per molecule of compound **52** and **51**. Based on the proposed structures, compound **52** and **51** would be anticipated to have 2 peroxide equivalents per molecule.

4.6. Extinction coefficient (ε)

An average extinction coefficient for SECO **2** standard at different concentrations was 3208 ± 0.035 (L mol^{-1} cm^{-1}) (Table 4.13). Compound **48** and compound **49** had extinction coefficients of 6413 and 6412 (L mol^{-1} cm^{-1}) respectively, which was double the extinction coefficient of SECO **2** (Table 4.14). Compounds **50** and **51**, had extinction coefficients of 3694 and 3795 (L mol^{-1} cm^{-1}) respectively, that were also higher than that observed for SECO **2**. Compound **52** had an extinction coefficient of 3209 (L mol^{-1} cm^{-1}), which was similar to SECO **2** (Table 4.14). Compounds **53** and **54** had ε=586, which was similar to the extinction coefficient of AAPH **47** after 5 h at 60°C (ε=586).

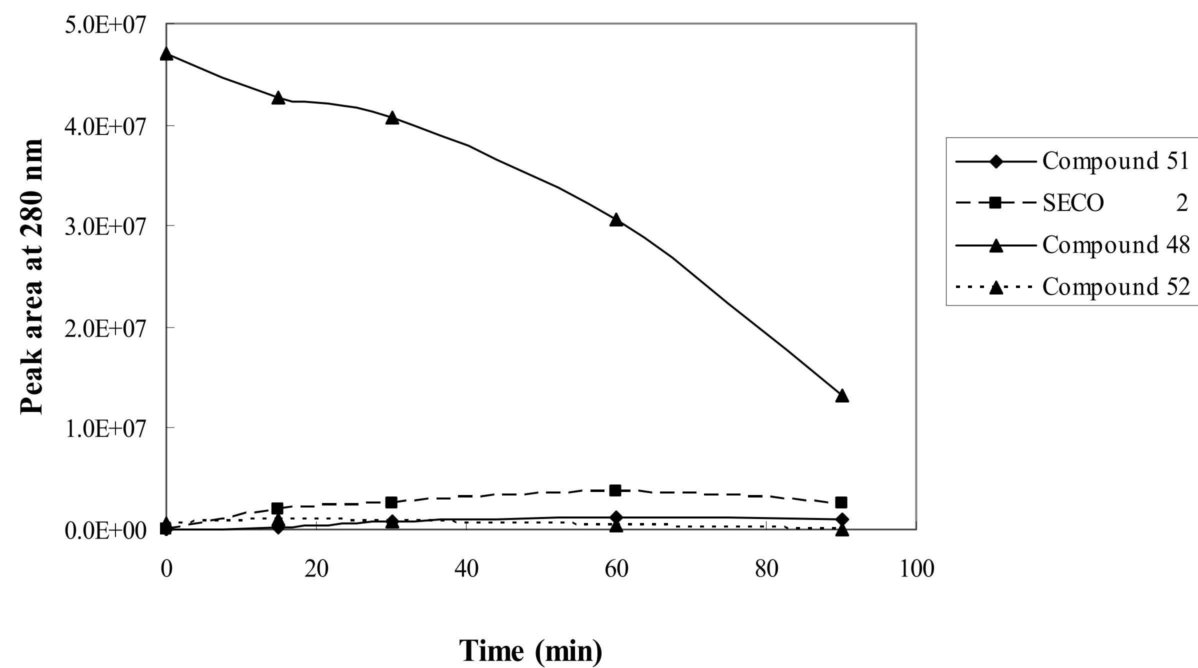


Figure 4.14a* Oxidation of compound **48** to SECO **2** and compounds **52** and **51** after reaction with AAPH **47** at 60°C for 30 min.

*4.14a shows non-expanded peak areas of oxidation compounds.

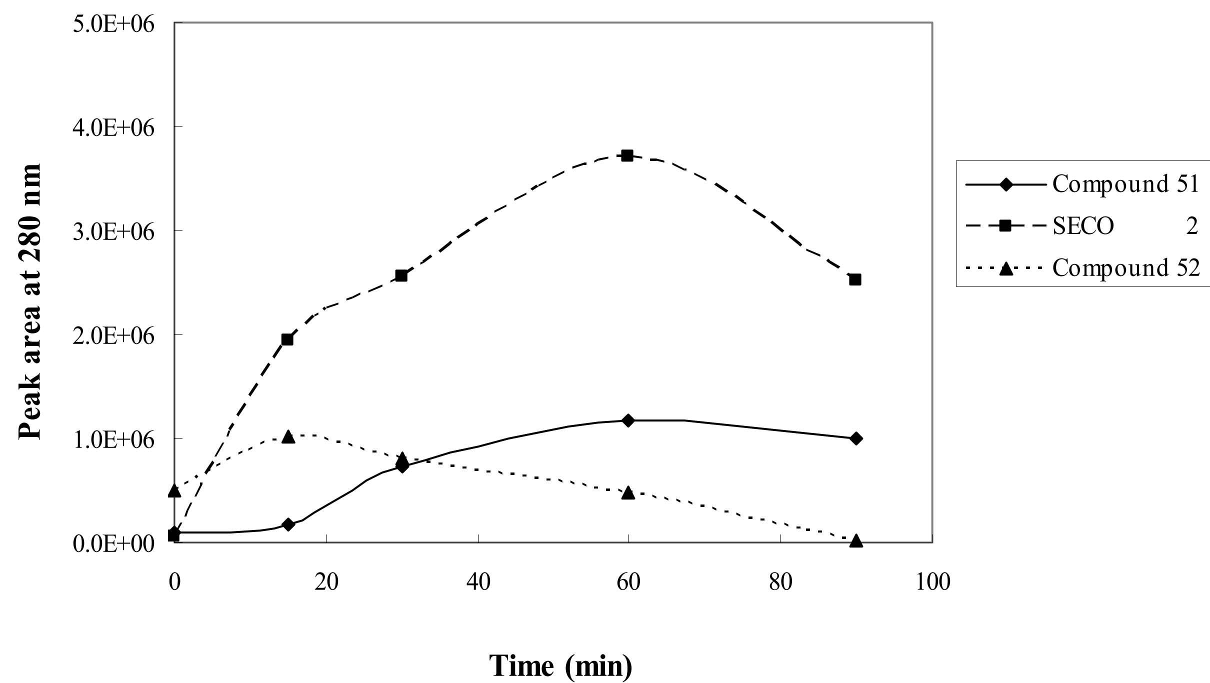


Figure 4.14b*. Oxidation of compound **48** to SECO **2** and compounds **52** and **51** after reaction with AAPH **47** at 60°C for 30 min.

*For simplicity, 4.14b shows expanded peak areas of oxidation compounds.

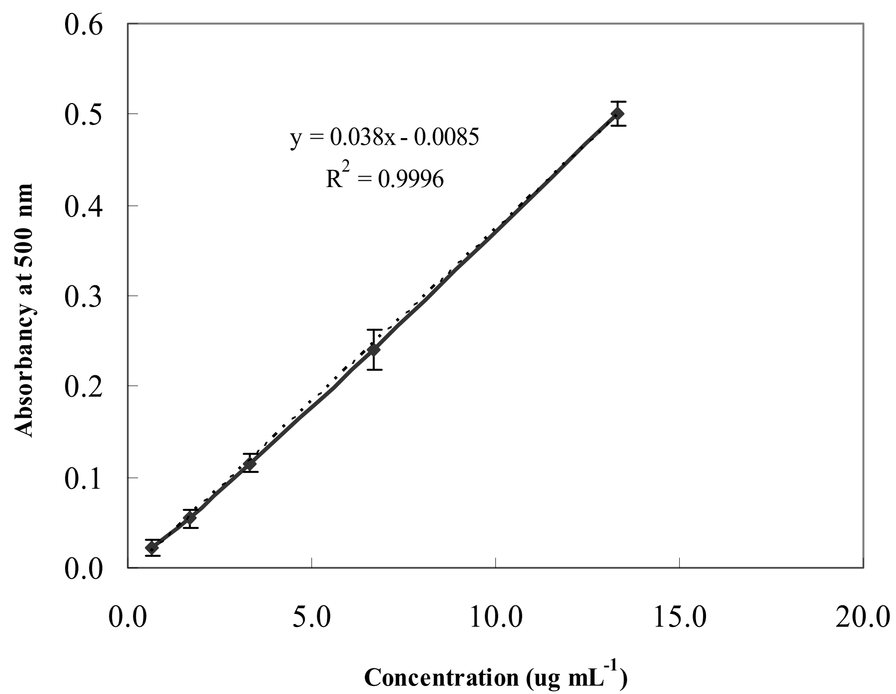


Figure 4.15. Mean (±SD) peroxide value for hydrogen peroxide μg mL^{-1} at 500 nm, n=4.

Table 4.13. Mean (±SD) extinction coefficient (L mol^{-1} cm^{-1}) for a SECO **2** standard (in methanol) at different concentrations, at 280 nm (n=5).

SECO 2 standard (mole/L)	**ε (L $mole^{-1}$ cm^{-1})**
0.001	3208 ± 0.021
0.002	3212 ± 0.042
0.004	3207 ± 0.082
0.006	3201 ± 0.009
0.008	3210 ± 0.021
0.01	3210 ± 0.095
Average	3208 ± 0.035

Table 4.14. Mean (±SD) extinction coefficients (L mol^{-1} cm^{-1}) of AAPH **47**-mediated SECO **2** oxidation products (in methanol), at 280 nm (n=5).

Compound	**mole/L**	**ε (L $mole^{-1}$ cm^{-1})**
48	0.0030	6413 ± 0.0302
49	0.0042	6412 ± 0.0460
50	0.0050	3694 ± 0.0719
51	0.0023	3795 ± 0.0006
52	0.0045	3209 ± 0.0515
53-54	0.012	586 ± 0.00030
AAPH **47** (5 h at 60°C)	0.010	586 ± 0.00075

4.7. Time-course of an AAPH 47-mediated oxidation of SDG 1

For SDG **1**, the same oxidation reaction conditions were applied as those used for SECO **2**. Oxidation over time of SDG **1** (0.01 mol L^{-1}) by AAPH **47** (0.1 mol L^{-1}) produced four major compounds **55** to **58**. Compounds, **56** and **57,** more polar than SDG **1** and compounds **55** and **58** less polar than SDG **1** were generated (Table 4.15).

The time-course indicated that compounds **55** and **58** were formed early and decomposed after 50 min while compounds **56** and **57** formed later in the reaction (Figure 4.16a). Control reactions were performed in the absence of AAPH **47** or SDG **1**. In the absence of AAPH **47**, no oxidation products were observed from SDG **1**. In the absence of SECO **2**/SDG **1**, AAPH **47** formed two major oxidation compounds that was discussed earlier (section 4.2).

The formation of SDG **1** oxidation compounds was time dependent. Compound **55** appeared in the early stages of the oxidation reaction, then declined after 50 min (Figure 4.16b). At this time, the formation of compounds **56** and **57** appeared to increase. Among all of the oxidation compounds, compound **56** was the major compound which, formed after 30 min, increased to the maximum level after 180 min and then decreased after this time. Compound **57** formed gradually and continued to accumulate until the reaction was terminated at (4 h). Compound **58** was the minor compound, detected after 120 min and decreased after 180 min. Semi-preparative HPLC, ES-MS and NMR were used to purify, isolate and identify these products.

Table 4.15. Oxidation compounds of SDG **1** (0.01 mol/L) in the presence of AAPH **47** (0.1 mol/L) at 60°C after 4h.

Compound	**Trivial Name**	**[a]RT (min)**	**[b]MW**	**UV-spectrum (nm)**
Compound **1**	SDG	13.3	686.6	205.7, 279.8, 224.7 [sh]
Compound **55**	5,5″-SDG dimer	14.3	1370	202.3, 280.6, 223.3 [sh]
Compound **56**	4,4″-AP-SDG	12.9	722.6	205.7, 279.8, 223 [sh]
Compound **57**	4,4″-SDG peroxide	12.1	718.4	202.2, 269.1, 351[sh]
Compound **58**	4-SDG-quinone	17.0	702.0	202.2, 276.2, 228.6 [sh]

[a]RT, retention time
[b]MW, molecular weight, calculated from mass spectral data.
[sh] shoulder

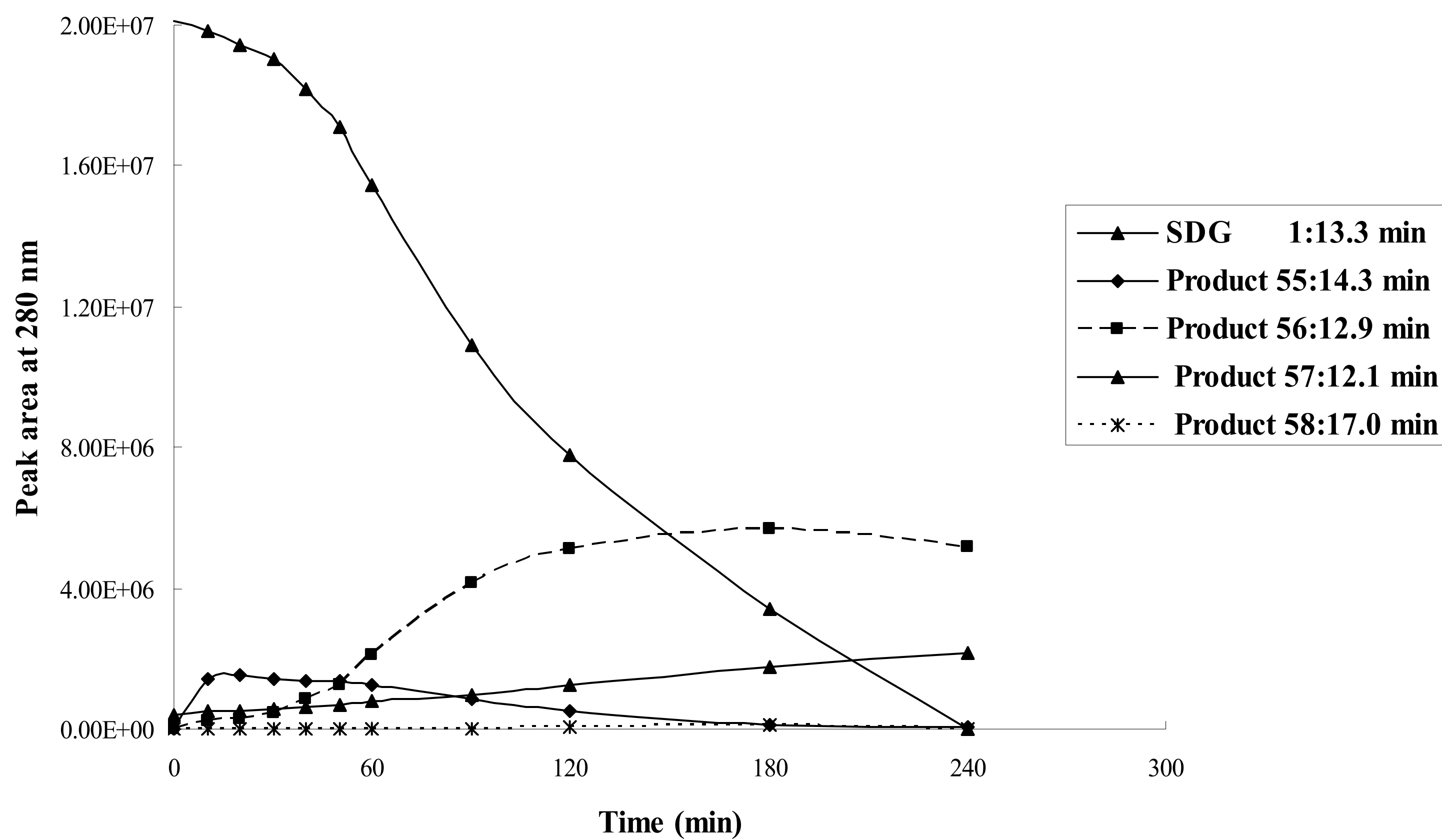


Figure 4.16a* Time course oxidation of SDG **1** (0.01 mol/L) by AAPH **47** (0.1 mol/L) at 60°C after 4h.
*16a shows non-expanded peak areas of oxidation products.

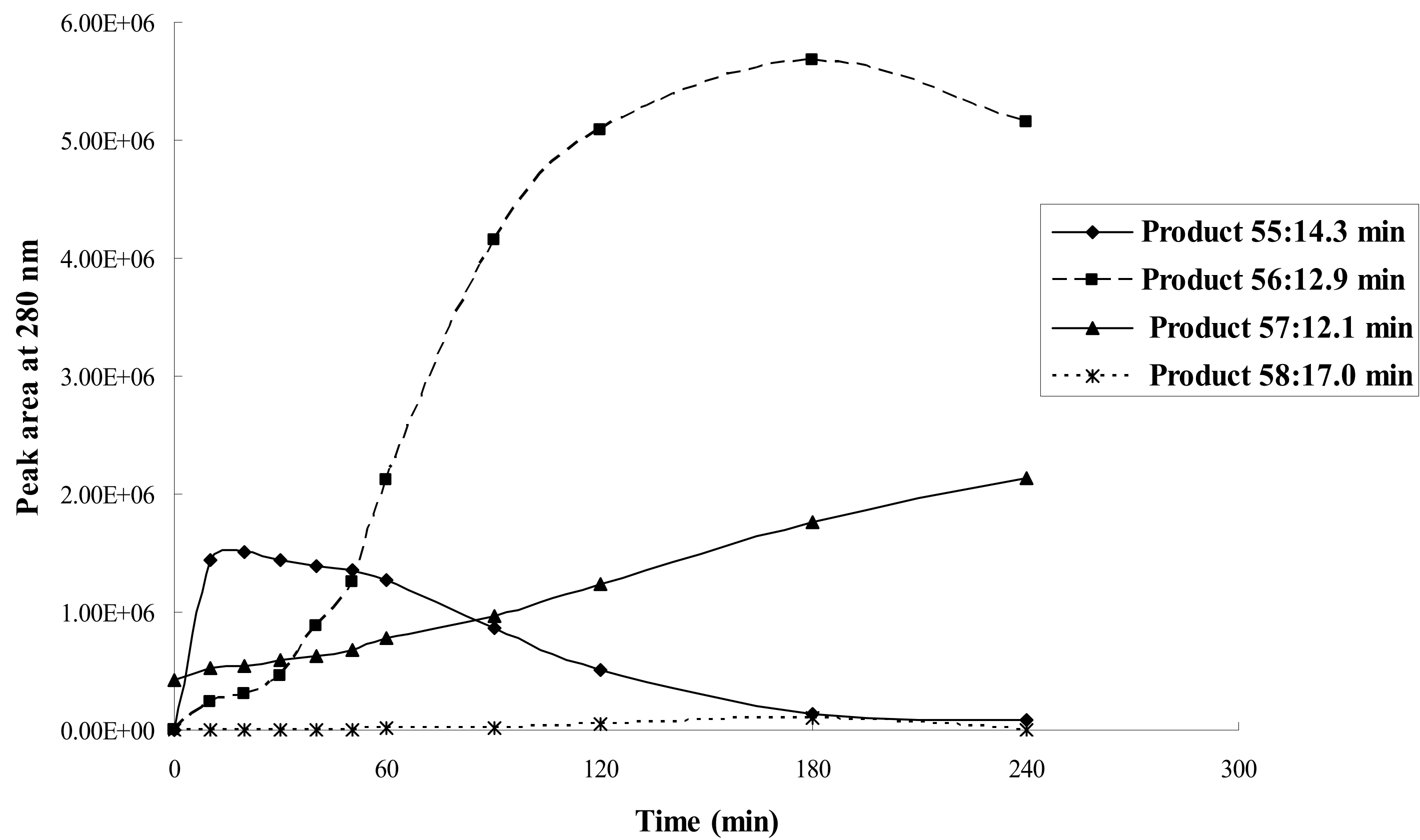


Figure 4.16b*. Time course oxidation of SDG **1** (0.01 mol/L) by AAPH **47** (0.1 mol/L) at 60°C after 4h.
*16b shows expanded peak areas of oxidation products.

4.8. Product yield of an AAPH 47-mediated oxidation of SDG 1

SDG **1** was oxidized by AAPH **47** to produce a total of 740 mg of four major oxidation compounds **55, 56, 57** and **58** (Table 4.16). The oxidation reaction also formed other minor compounds, which were not identified (Table 4.16). After the 4h oxidation reaction 220 mg of SDG **1** was recovered. Overall, 390 mg of SDG **1**-containing oxidation reaction materials were lost during purification. The UV-detectable profile by HPLC for oxidation of SDG **1** by AAPH **47** is illustrated in Figure 4.17.

4.9. Structural determination

4.9.1. SDG 1

Table 4.17, shows ^{1}H-NMR and ^{13}C-NMR assignments for SDG **1**. These data are similar to those reported previously.[24,29,285] SDG **1** had RT=13.3 min from an HPLC experiment and showed a $[M+H]^+$ peak at *m/z*=687.6 in ES-MS in positive mode corresponding to the molecular formula $C_{32}H_{46}O_{16}$. The ^{1}H NMR spectrum exhibited an ABC (three spin-coupled systems) for the aromatic protons and the methoxy singlet at δ 3.73, a series of resolved multiplets extending from δ 4.24 to 2.12 for a total of 18 non-exchangeable protons (Table 4.17). The presence of five hydroxyl groups, gives a total of 23 hydrogen atoms, thus confirming that **1** is a symmetrical molecule. The mass spectrum displayed a pseudo-molecular ion peak at *m/z* 687.6 $[M+H]^+$, consistent with the mass of singly protonated SDG **1** supporting presence of a dimeric glucosylated phenyl propanoid.

Table 4.16. Yield of oxidation compounds from SDG **1** (0.01 mol L^{-1}) by AAPH **47** (0.1mol L^{-1}) for 4 h at 60°C.

Oxidation Product	**Yield (mg)**	**Yield %**
Compound **55**	135.5	9
Compound **56**	305.0	10
Compound **57**	119.4	8
Compound **58**	180.1	10.5
Total major compounds	740.0	37.5
Total minor compounds	650.0	32.1
Unoxidized SDG **1**	220.0	11.3
Overall loss	390.0	19.1

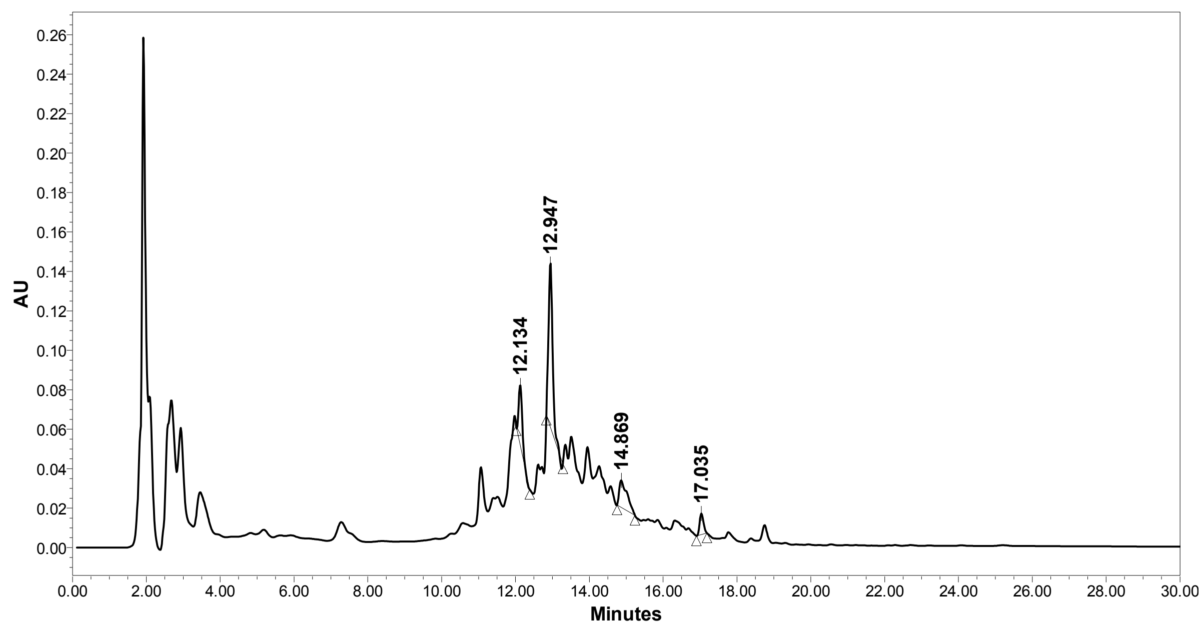
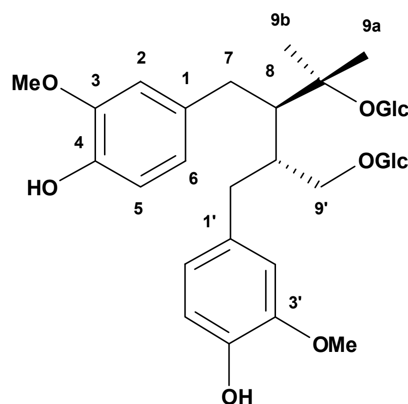


Figure 4.17. HPLC chromatogram of oxidation reaction of SDG **1** by AAPH **47** at 60°C after 4 h, λmax at 280 nm.



SDG **1**
$C_{32}H_{46}O_{16}$

Table 4.17. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of SDG **1** in CD_3OD.

Position	**SDG 1**		
	δ_C	δ_H **(multiplicity,** ***J*** **Hz)**	**HMBC**
1/1′	133.9	-	5, 9a/9b, 8/5′, 9′a/9′b, 8′
2/2′	113.5	6.58 (d, 2.0)	6, 7a/7b/6′, 7′a/7′b
3/3′	148.7	-	5, 2, OCH3/5′, 2′, OCH3
4/4′	145.4	-	5, 2, 6/5′, 2′, 6′
5/5′	115.7	6.64 (d, 8.0)	-
6/6′	122.9	6.56 (dd, 2.0, 8.0)	7a/7b, 2
7a/7′a	35.6	2.61 (dd, 8.2, 13.7)	2, 6, 8, 9a/9b
7b/7′b	35.6	2.68 (dd, 6.5, 13.7)	2′, 6′, 8′, 9′a/9′b
8/8′	41.2	2.12 (t, 6.0)	9a/9b, 7a/7b/9′a/9′b, 7′a/7′b
9b/9′b	71.2	3.47 (m, 6.5, 9.8)	9-O-Glc (anomeric), 8, 7a/7b
9a/9′a	71.2	4.08 (dd, 5.5, 9.8)	9′-O-Glc (anomeric), 8, 7′a/7′b
OCH_3	56.3	3.73 (s)	-
9-O-Glc (anomeric)	104.8	4.24 (d, 7.7)	9a/9b, Glc
9-O-Glc	62.8-78.2	3.10-3.90 (m)	Glc (anomeric), 9a/9b

A DEPT experiment together with 2D-NMR experiments including COSY, HMQC, and HMBC, confirmed the presence of three methylenes, nine methines, one methyl and three quaternary carbons for a total of 16 signals (Table 4.17). From the chemical shift, the quaternary carbons are easily attributed to the tri - substituted aromatic ring. The distinction between C-3/3′ and C-4/4′ was achieved on the basis of the ^{3}J (C,H) coupling of the former with the methoxy protons. In addition, COSY and HMQC spectra verified an ABC spin system, together with an isolated methoxy group, which corresponds to the protons of the methylenes and/or methines of C-9, C-8, C-7, the glucose moiety and the aromatic system.

For the glucose portion of SDG **1**, analysis of COSY spectrum starting from the anomeric proton at δ 4.24 assigned by its ^{1}H shift and from HMQC connectivity led to present of multiplets in the one dimentional spectrum. The coupling constants unambiguously provided the identification of the carbohydrate residue in the structure (Table 4.17). When NOESY spectra were recorded for SDG **1** to check the appearance of cross peaks, meaningful differences in the intensities of NOE suggested the existence of preferred conformations of H-9/9′. These chemical shifts were significantly different for the methylene protons of the 9/9′-CH_2O. The integrals of the corresponding NOE cross-peaks in the NOESY spectrum clearly indicated that the proton with the larger δ (4.08) at H-9a is closer to the anomeric proton than H-9b and stereochemical differentiation is then possible: H-9a is pro-R and H-9b is pro-S.

4.9.2. Identification of compound 55 (5,5”-SDG dimer)

The ES-MS data for compound **55** (RT=14.5 min) (Table 4.18) displayed a pseudo-molecular ion peak at *m/z* 1371 $[M+H]^+$ in positive mode, which is consistent with a dimer of SDG **1** (Figure 4.18). The molecular formula of **55** was then calculated to be $C_{64}H_{90}O_{32}$. The ^{13}C NMR (Table 4.18) spectrum displayed signals for 32 carbon atoms suggesting that compound **55** was a symmetrical molecule (Figure 4.18). Chemical shifts of the aliphatic and that of one of the aromatic ring moieties were very similar to those of the corresponding carbons in SDG **1**. Important differences were observed for C-5/5″ (126.9) and C-6/6″ (125.4) compared to 115.7 and 122.9 for the same carbons in SDG **1**. This suggested that these two SDG **1** molecules were linked via their aromatic rings. The ^{1}H NMR spectrum for **55** showed an ABC system [6.58 (d, 1.5), 6.64 (d, 8), and 6.57 (d, 1.5, 8.0)] and an AB [δ 6.65 and 6.61 (1H each d, 1.5)] system for aromatic rings (Table 4.18). In the HMBC spectrum, correlation peaks observed between H-6/H-6″ (δ 6.61) to C-1/C-1″, C-4/C-4″, C-5/C-5″ and C-7/C-7″ established a connection from C-5 to C-5″ for dimerization (Table 4.18). In addition, COSY and HMQC spectra of compound **55** verified an ABC spin system, together with an isolated methoxy group, that corresponds to the protons of the methylenes of C-9, C-8 and methines of C-7. Compound **55** would be predicted to have *n*=1 due to the loss of one hydrogen from SDG **1** that undergoes dimerization (section 5.6).

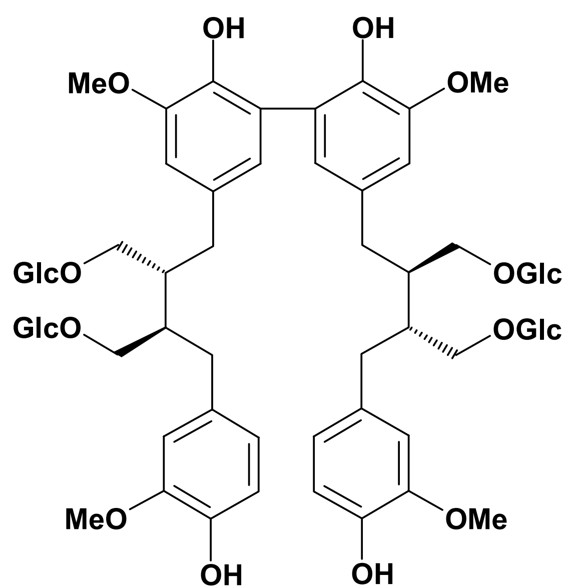


Figure 4.18. Proposed structure for compound **55**.

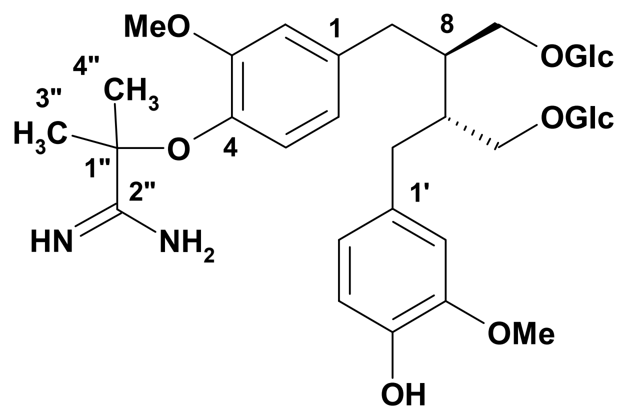
Table 4.18. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of **55** in CD_3OD.

	Compound 55	
Position	δ_C	δ_H **(multiplicity, *J* Hz)**
1,1″	134.0	-
2,2″	115.6	6.65 (d, 1.5)
3,3″	149.3	-
4,4″	146.5	-
5,5″	126.9	-
6,6″	125.4	6.61 (d, 1.5)
7,7″	35.6	2.63 (m)
8,8″	41.3	2.10 (br t, 6.1)
9a, 9a′, 9a″, 9a‴	71.2	4.04 (m, 5.5, 9.8)
9-Clc	62.8-78.2	3.10-3.90 (m)
9-O Glc (anomeric)	104.7	4.28 (d, 7.7)
3, 3″-OCH_3	56.5	3.73 (s)
1′, 1‴	134.0	-
2′,2‴	113.6	6.58 (d, 1.5)
3′,3‴	148.8	-
4′,4‴	145.4	-
5′,5‴	115.8	6.64 (d, 8.0)
6′,6‴	122.9	6.57 (dd, 1.5, 8.0)
7′,7‴	35.5	2.61 (m)
8′,8‴	41.3	2.10 (t, 6.1)
9b, 9b′, 9b″, 9b‴	71.2	3.43 (m, 6.5, 9.8)
9-Clc	62.8-78.2	3.10-3.90 (m)
9-O Glc (anomeric)	104.7	4.24 (d, 7.7)
3′, 3‴-OCH_3	56.3	3.71 (s)

4.9.3. Identification of compound 56 (4,4”-AP-SDG)

The ES-MS data of **56** (RT=12.9 min) showed pseudo-molecular ion peaks at *m/z* 772 $[M+H]^+$ and 770 $[M-H]^-$ in positive and negative mode (Table 4.19), respectively, suggesting the addition of one AP radical to the SDG **1** molecule (Figure 4.19). The molecular formula for **56** was suggested by combined analysis of ES-MS, 1D- and 2D-NMR data. Molecular formula of **56** was then calculated to be $C_{36}H_{54}O_{16}N_2$. The structure of compound **56** is shown in Figure 4.19.

The ^{13}C NMR spectrum showed that **56** was not a symmetrical molecule by displaying signals for 36 carbon atoms (Figure 4.19). Signals from an AP moiety were found at δ 176.5, 80.1, and 28.0. A large singlet for methyl groups was observed at δ 1.51, plus two close but distinguishable ABC systems for the aromatic protons. There was no correlation between SDG **1** and AP moieties in the HMBC spectrum suggesting that AP is linked to the aromatic group at the 4-OH position. The AP carbon chemical shift at δ 80.1 (s) was an indication of its linkage to oxygen. The changes observed from the chemical shift of C-1 (140.7), C-3 (154.0), C-4 (146.1), and C-5 (124.9) in **56** compared to 133.9, 148.7, 145.4, and 115.7 for the same carbons in SDG **1** could be due to an AP moiety on the 4-OH. Like compound **50** from SECO **2** oxidation, compounds **56** from SDG **1** oxidation would be predicted to have *n*=2 due to the loss of one hydrogen and trapping an AP radical (section 5.6).



56

Figure 4.19. Proposed structure for compound **56**.

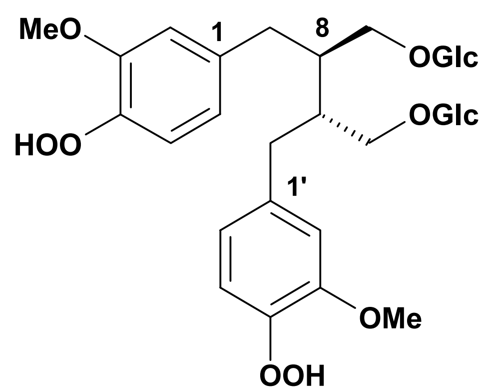
Table 4.19. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of **56** in CD_3OD.

		Compound 56			
Position	**δ_C**	**δ_H (multiplicity, J Hz)**	**Position**	**δ_C**	**δ_H (multiplicity, J Hz)**
1	140.7	-	1′	133.9	-
2	114.6	6.73 (brs)	2′	113.8	6.65 (br s)
3	154.0	-	3′	148.6	-
4	146.1	-	4′	145.3	-
5	124.9	6.91 (d, 8.0)	5′	114.1	6.64 (d, 7.9)
6	124.8	6.67 (br d, 8.2)	6′	122.7	6.51 (br d, 8.0)
7	35.8	2.68 (m)	7′	35.5	2.63 (m)
8	41.4	1.91 (br s)	8′	41.2	1.96 (br s)
9	70.7	3.59 (m)	9′	71.0	3.61 (m, 4.5, 10.5)
3-OCH_3	56.0	3.73 (s)	3′-OCH_3	56.1	3.69 (s)
9-O-Glc	104.6		9′-O-Glc	104.4	
9-O-Glc	62.7-78.1		9′-O-Glc	62.7-78.1	
			1″	80.1	-
			2″	176.5	-
			3″/4″	28.0	1.56 (s)

4.9.4. Identification of compound 57 (4,4"-SDG-peroxide)

The ^{1}H- and ^{13}C-NMR data for **57** (RT=12.1 min) were similar to those of SDG **1** (RT=13.3 min) displaying signals for only half of the molecule (Table 4.20). The difference between **57** and **1** was suggested based on their RT and ES-MS results.

In the ES-MS, the molecular ion peak of **57** (RT=12.1 min) appeared at *m/z* 717 $[M-H]^-$ in negative mode suggesting addition of two oxygen atoms to SDG **1**. The molecular formula for **57** was calculated to be $C_{32}H_{46}O_{18}$. NMR experiments revealed changes in the chemical shifts of, C-2 (114.8), C-3 (149.7), C-4 (149.4) and C-5 (117.1) in (**57**) compared to 113.5, 148.7, 145.4 and 115.7 for the same carbons in SDG **1** (Table 4.17). In addition, ^{1}H NMR spectra verified an ABC spin system, together with an isolated methoxy group, which corresponds to the protons of the methylenes at C-9 and C-7 and methines of C-8, the glycosidic moiety and the aromatic system. The glucose portion was assigned by its ^{1}H chemical shift and from COSY and HMBC correlations. The coupling constants unambiguously provided the identification of the carbohydrate residue in the structure and indicated that the glucose moieties were unchanged (Figure 4.20). Compound **57** would predict to have *n*=0 because it does not lose any protons or trapping any AP free radicals (section 5.6). Compound **55** would be predicted to have *n*=1 due to the loss of one hydrogen from SDG **1** molecule that undergoes dimerization. Like compound **50** from SECO **2** oxidation, compounds **56** from SDG **1** oxidation would have *n*=2 due to losing one hydrogen and trapping an AP radical.



57

Figure 4.20. Proposed structure for compound **57**.

Table 4.20. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of **57** in CD_3OD.

Position	Compound 57		
	δ_C	δ_H (multiplicity, *J* Hz)	HMBC
1/1′	133.9	-	5, 9a/9b, 8/5′, 9′a/9′b, 8′
2/2′	114.8	6.57 (d, 2.0)	6, 7a/7b/6′, 7′a/7′b
3/3′	149.7	-	5, 2, OCH3/5′, 2′, OCH3
4/4′	149.4	-	5, 2, 6/5′, 2′, 6′
5/5′	117.1	6.63 (d, 8.0)	-
6/6′	123.1	6.54 (dd, 2.0, 8.0)	7a/7b, 2
7a/7′a	35.3	2.59 (dd, 8.3, 13.7)	2, 6, 8, 9a/9b
7b/7′b	35.6	2.66 (dd, 6.5, 13.7)	2′, 6′, 8′, 9′a/9′b
8/8′	41.2	2.11 (t, 5.6)	9a/9b, 7a/7b/9′a/9′b, 7′a/7′b
9a/9′a	71.2	3.46 (m, 6.4, 9.8)	9-O-Glc (anomeric), 8, 7a/7b
9b/9′b	71.2	4.05 (dd, 5.4, 9.8)	9′-O-Glc (anomeric), 8, 7′a/7′b
OCH_3	56.3	3.71 (s)	-
9-O-Glc	104.7	4.21 (d, 7.7)	9a/9b, Glc
9-O-Glc	62.8-78.2	3.10-3.90	Glc (anomeric), 9a/9b

4.9.5. Identification of compound 58 (4-SDG quinone)

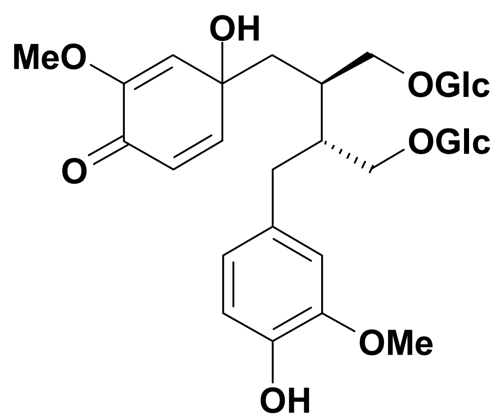
The ES-MS analysis for compound **58** (RT=17.0) showed the molecular ion peak at *m/z* 701 $[M-H^-]^-$, suggesting a molecular formula of $C_{32}H_{46}O_{17}$ for **58** (Table 4.15). The ^1H NMR spectra showed an ABC system of aromatic protons [^1H δ 6.65 (d, 2) and 6.91 (d, 10.0) and 6.60 (d, 8.2)]. This was an indication that one of the aromatic rings in SDG **1** had been oxidized (Figure 4.21).

The ^{13}C NMR for compound **58** displayed signals for a carbonyl carbon at δ 171.0 plus an oxygenated carbon at δ 73.4 (Table 4.21) which are not seen for SDG **1** (Table 4.17). DEPT, COSY, HMQC, and HMBC experiments confirmed the presence of three methylenes, nine methines, one methyl and three quaternary carbons for a total of 16 signals (Figure 4.21). In addition, ^1H NMR and ^{13}C spectras verified an isolated methoxy group (Figure 4.21). Compound **58** would have *n*=2 due to first, losing hydrogen for the formation of a double bond (*n*=1) and, then, the addition of hydrogen (*n*=1) for the formation of a quinone molecule (section 5.6).

4.10. Confirmation tests for compounds 55 and 57

4.10.1. Oxidation of product 55

Further oxidation of compound **55** with AAPH **47** showed the formation of SDG **1** and then conversion of SDG **1** to compound **56** (Figure 4.22).



58

Figure 4.21. Proposed structure for compound **58**.

Table 4.21. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of compound **58** in CD_3OD**.**

		Compound 58			
Position	δ_C	δ_H **(multiplicity,** ***J*** **Hz)**	**Position**	δ_C	δ_H **(multiplicity,** ***J*** **Hz)**
1	73.4	-	1′	133.9	-
2	137.6	6.65 (d, 2)	2′	113.5	6.61 (d, 2.0)
3	150.5	-	3′	147.4	-
4	171.0	-	4′	146.3	-
5	127.6	6.91 (d, 10)	5′	115.4	6.64 (d, 7.9)
6	129.7	6.60 (br d, 8.2)	6′	122.9	6.54 (br d, 8.0)
7	37.6	2.67 (m)	7′	35.6	2.61 (m)
8	42.2	2.25 (br s)	8′	41.2	2.11 (br s)
9	71.6	3.59 (m)	9′	71.2	3.61 (m, 4.5, 10.5)
3-OCH_3	56.9	3.74 (s)	3′-OCH_3	56.7	3.72 (s)
9-O-Glc (anomeric)	104.8	4.04 (m)	9′-O-Glc	104.8	4.23 (m)
9-O-Glc	62.3-78.2	3.11-3.91	9′-O-Glc	62.5-78.2	3.11-3.91

4.10.2. Peroxide value of compound 57

The peroxide value test confirmed the presence of a small quantity of peroxide (0.85 μg mL^{-1}) in compound **57** (calculated from Figure 4.15). This was equivalent to a total number of 1.7 ± 0.19 peroxide equivalents per molecule of compound **57**. Compound **57** would be anticipated to have 2 peroxide equivalents per molecule.

4.11. Extinction coefficient

Average extinction coefficient (L mol^{-1} cm^{-1}) of SDG **1** standard was 3204 (Table 4.22). In Table 4.23, extinction coefficient (L mol^{-1} cm^{-1}) of the major AAPH **47**-mediated SDG **1** oxidation compounds is shown. The extinction coefficient was used to estimate the concentration based on the data from the HPLC chromatogram (Table 4.23). For this calculation, the maximum UV-absorption (280 nm) observed from HPLC was used. These results were similar to the calculated concentration from mass (Table 4.23). Compound **55** had an extinction coefficient of 6405 ± 0.0037 (Table 4.23), which was almost twice the extinction coefficient of SDG **1**. Compound **56** had an extinction coefficient of 3765 ± 0.0089, which was higher than the extinction coefficient of SDG **1**. Compounds **57** and **58** had extinction coefficients of 3208 ± 0.0103 and 3209 ± 0.0049, respectively, which were similar to the extinction coefficient of SDG **1**.

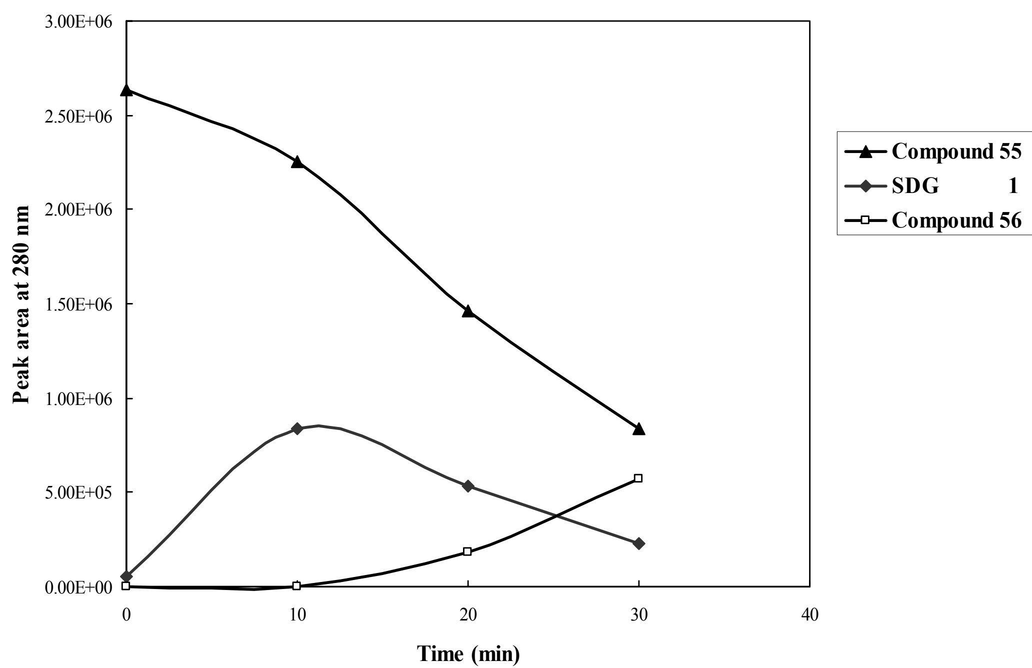


Figure 4.22. AAPH **47**-mediated oxidation of compound **55** to SDG **1** and compound **56** (60°C for 30 min).

Table 4.22. Mean (±SD) extinction coefficient (L mol^{-1} cm^{-1}) of SDG **1** standard (in methanol) at different concentrations, at 280 nm (n=5).

Conc (mole/L)	Extinction coefficient (L mol^{-1} cm^{-1})
0.001	3200 ± 0.0010
0.002	3206 ± 0.0020
0.004	3203 ± 0.0040
0.006	3204 ± 0.0060
0.008	3203 ± 0.0080
0.01	3207 ± 0.0100
Average	3204 ± 0.0052

Table 4.23. Mean (±SD) extinction (L mol^{-1} cm^{-1}) of SDG **1** oxidation compounds with AAPH **47** (in methanol), at 280 nm (n=5).

Compound	Conc (mole/L) from mass	Conc (mole/L) from HPLC (UV data)	Extinction coefficient (L mol^{-1} cm^{-1})
55	0.000988	0.000956	6405 ± 0.0037
56	0.003956	0.003553	3765 ± 0.0089
57	0.001663	0.001610	3208 ± 0.0103
58	0.002564	0.002279	3209 ± 0.0049
AAPH **47** after heat	0.010000	0.010000	586 ± 0.00030

5.0. DISCUSSION

5.1. SECO and SDG as chain-breaking antioxidants

The major objectives of this study were to investigate the antioxidant properties and major oxidative compounds of SECO **2** and SDG **1** using an AAPH **47** *in vitro* model of lipid peroxidation. This investigation was facilitated by the structural elucidation of the major oxidative compounds from the reaction with AAPH **47** and the stoichiometric ratio determination of SECO **2** and SDG **1** compared to BHT **17,** as a positive control. This study relates the results of the product study to the relative antioxidant capacity. Finally, the antioxidant capacity was confirmed using two different systems. In addition, this research confirmed the antioxidant activity of flaxseed lignans and provided an insight into the antioxidant chemistry.

Antioxidant food preservatives such as BHT **17** are commonly used to minimize autoxidation of oil-based food products,[223,275] typically at levels of 75 mg kg^{-1} in foods.[279,280] Concern has arisen regarding potential liver toxicity from chronic use of these compounds.[276] This has lead to a growing interest in finding alternative antioxidant food preservatives, specifically natural products that may also have the potential to provide additional health benefits. Flaxseed lignans are natural antioxidants with no known toxicity and may have potential health benefits associated with their consumption.[6,124,127,151,274]

Based on the chemical structure, SECO **2** is expected to be a more lipophilic antioxidant than SDG **1**. The present study demonstrated that in a liposomal system, SECO **2** (on a molar basis) has a higher free-radical trapping capacity than does SDG **1**. This higher capacity is attributed to the aglycone structure of SECO **2** (compared to SDG **1**, a glycoside) providing greater solubility in liposomes. A similar observation has been previously reported for the flavonoid quercetin which was shown to have a higher antioxidant activity than its glycoside, rutin.[165,286,287] SDG **1** has been reported to prevent the development of disease related to oxidative stress,[119,127,130] these results suggest SECO **2** may also be effective in the prevention of oxidative stress by free-radical scavenging and prevention of oxidation.[154,288]

The results reported here indicate that flaxseed lignans may be useful as natural antioxidant preservatives and might be applicable in this role in vegetable oils and other foods. Both *in vitro* and *in vivo* studies on human and animal models demonstrate the health benefits of flaxseed lignans, including decreases in breast and prostate cancers, delayed onset of diabetes, protection against lupus-induced nephritis, bone loss and heart disease.[129,277,289] It may also be attractive to include flaxseed lignans as a preservative in vegetable oil-based products because of the potential to provide additional health benefits as antioxidant or weak estrogenic/anti-estrogenic compounds.[50,68,133,135,290] The requirement for increased amounts of flaxseed lignans for this purpose could also provide a value-added economic benefit to flaxseed growers.

The conclusions from the data for the *in vitro* oxidation systems cannot be extended directly to the *in vivo* oxidation system. The *in vivo* system involves complex processes and feedback mechanisms that cannot be reproduced using *in vitro* experiments.[279,280] Many reactions take place competitively *in vivo* and the overall antioxidant activities are determined by a delicate balance of these competing reactions. Nevertheless, the behaviour of antioxidants *in vivo* are unlikely to be understood unless the chemistry and biochemistry of these antioxidants for the *in vitro* systems are elucidated.[279,280]

5.2. Antioxidant capacity of flaxseed lignans in liposomal and vegetable oil systems

The ability of flaxseed lignans to delay the onset of oxidation in two model systems was examined. These studies demonstrated the potential use of flaxseed lignans as naturally-occurring food preservative antioxidants. First, a peroxyl radical-mediated liposomal oxidation system was used to calculate an antioxidant stoichiometric value which measured the number of radicals consumed per molecule of antioxidant.[149,230] Second, the ability of each antioxidant was examined to find whether they can inhibit rancidity in a commercially available vegetable oil via measurement of lipid oxidation levels using a Rancimat analyzer.[279,280]

Dietary antioxidants broadly include radical scavengers, metal chelators, oxidative enzyme inhibitors, and antioxidant enzyme cofactors.[150,230] A variety of

tests have been developed to express antioxidant potency. These tests can be categorized into two groups:

1) Assays for radical scavenging ability.[150,230,233] Some of the commonly used methods include: measuring the reduction of stable radicals (DPPH radicals), [291] measuring the ability to scavenge radicals generated using azo-initiators (AAPH, AMVN and ABTS), [292] deoxyribose assay for non site-specific and site-specific Fenton reactant-induced •OH scavenging activity[151,231], and scavenging of hydroxyl radical generated in the FRBR assay.[230]

2) Assays that test the ability to inhibit lipid oxidation under accelerated conditions.[232] Accelerated test systems mainly include lipids, which rapidly oxidize in order to simulate a long induction period in a short time. To accelerate oxidation, an increase in temperature is often used.[150,232] Both the stoichiometric ratio and Rancimat methods reported here were based on lipid oxidation under thermal acceleration.

5.2.1. Stoichiometric ratio

The purpose of this study was to quantitate the ability of the flaxseed lignans to inhibit lipid peroxidation, thus necessitating model systems that mimic lipid peroxidation. The stoichiometric ratio uses liposomes as a mimic of *in vivo* lipid peroxidation. Thermal decomposition of AAPH **47** induces autoxidation of linoleic acid, where phosphatidylcholine and linoleic acid form phospholipid liposomes when dispersed in water.[230] An antioxidant's ability to inhibit liposomal lipid

peroxidation is given a numerical value, the stoichiometric ratio *n*. This method relies on calibration with an antioxidant with a known reaction stoichiometry in this system. In this case BHT **17**, which reacts with two peroxyl radicals per molecule (*n*=2) was chosen. It has been generally observed that the kinetics and mechanisms for the oxidation of lipids in liposomal membranes are essentially the same as those of phospholipids and fatty acids in organic solution.[230] Therefore, the liposomal system can serve as a good *in vitro* model for biological membranes. This study did not use an emulsifier as stabilizer since some emulsifiers (e.g. Tween) can increase free radical generation which may produce a pro-oxidant rather than inhibitory effect.[149,225]

The progress of autoxidation was monitored by the formation of primary oxidation products, conjugated dienes (240 nm).[150] In the absence of antioxidant (blank control), linear formation of conjugated dienes was observed, as described by the equation y=0.0425x + 0.2784. Addition of BHT **17**, SECO **2** or SDG **1** resulted in inhibition of conjugated diene formation as indicated by a lag phase where limited conjugated diene formation occurred. Since formation of conjugated diene products occurs during the chain propagation phase it would be expected that a non-zero level of conjugated dienes would be formed if the lignans are indeed quenching chain propagation. When all of the antioxidant was consumed the rate of formation of conjugated dienes returned nearly to the levels seen for the blank control. The slopes of the curve (*Ri* value or rate of oxidation) were inversely proportional to the concentration of SECO **2** and SDG **1**, which is consistent with values previously

reported for the oxidation of vitamin E and β-carotene.[278,293] The intersection of these two lines (inflection point) defines the lag phase (τ) which can be related to the stoichiometric ratio. Due to the gradual change in slope near the point of intersection, there is some uncertainty in determination of an exact inflection point. Therefore the selection of data points defining the two lines was biased in favour of early and late data points.

The stoichiometric ratio determination provided valuable information about the number of peroxyl radicals consumed per molecule of SECO **2** and SDG **1** as compared to the widely used synthetic antioxidant BHT **17**. The contribution of aromatic hydroxyl groups in oxidation reactions are well known for phenolic antioxidants.[279,280] It was anticipated that the *n*-value for SECO **2** and SDG **1** could be estimated from the relationship between *n*-value and the number of active hydroxyl groups in their structures.[227] SECO **2** and SDG **1** have an equal number of aromatic hydroxyl groups and therefore, they would be expected to scavenge an equal number of free radicals, with a maximum stoichiometric ratio of $n=4$ predicted for both. In this study SECO **2** and SDG **1** were determined to have different stoichiometric ratios and both possessed an $n<4$, less than might be expected from the number of their active hydroxyl groups, similar to those found by Roginsky (2003)[227] for catechol derivatives.

The stoichiometric ratio (1.5) of SECO **2** indicates that SECO **2** has a higher free-radical scavenging capacity than SDG **1** (1.1-1.2). The stoichiometric ratio values well below the ideal value of $n=4$ suggest that the predicted models for

flaxseed lignan antioxidant reactions do not accurately reflect the antioxidant process. The greater antioxidant capacity of SECO **2** is likely due to two possibilities, (i) greater solubility in the liposomal system of SECO **2** vs SDG **1**, (ii) involvement of the two alcoholic hydroxyl groups on SECO **2** that are glycosylated in SDG **1** in the antioxidant reactions. Consistent with these results are studies which have indicated that flavonoids and (-)-epicatechin with glycosylated phenolic groups show lower antioxidant activities than their aglycones.[251,294-296] Isoflavones in the aglycone form have higher antioxidant activities than their glycosides and it has been suggested that this is the result of increased lipophilicity.[226,287] Since the number of phenolic hydroxyl groups in the aglycone form of flavonoids, and catechins has an effect on their free-radical scavenging capacity, it is likely that the diminished antioxidant capacity in those glycosylated systems is the result of blocking key hydrogen donating phenol groups, although reduced lipid solubility may also play a role.[251,294-296]

In the case of SDG **1**, the hydroxyl groups which are blocked are alcohols rather than phenols and would not be expected to participate in the antioxidant reactions, suggesting reduced lipid solubility as a likely explanation. If the alcohols do contribute to the antioxidant reactions it is conceivable that the alcohols on the glucose moieties would also contribute to the antioxidant capacity. This would suggest that SDG **1** should have higher antioxidant capacity than SECO **2** which was not observed in the present study. The product studies in section 5.3 will address the reactivity of the alcohol groups on SECO **2**. Furthermore, a new proposed

antioxidant model for the flaxseed lignans based on the determination of the products from AAPH **47** mediated oxidation of SECO **2** and SDG **1** will be discussed in section 5.6.

Antioxidants are divided into two categories, preventive antioxidants and chain-breaking antioxidants.[226] The preventive antioxidants (e.g. glutathione peroxidase and catalase) deactivate the active species (e.g. H_2O_2) without further generation of free radicals and, thereby, reduce the rate of chain initiation.[226] Chain-breaking antioxidants have the ability to scavenge chain-propagating oxygen radicals to produce stable, non-radical products and suppress lipid peroxidation.[226] Therefore, the antioxidant capacity, *n*, shows how many chains may be terminated by one molecule of antioxidant.[226,227] BHT **17** traps peroxyl radicals to produce a stable phenoxyl radical or to form stable non-radical products.[197,228] Niki *et al.* (1985)[228] demonstrated that chain-breaking antioxidants, such as vitamin E, can scavenge the chain-propagating oxygen radicals generated from AMVN and suppress the peroxidation of liposomal membranes in aqueous dispersions.[226] Lee *et al.* (2004)[197] reported that cysteine and glutathione could also act as chain-breaking antioxidants by scavenging oxygen-centered radicals and suppressing the oxidation of lipids.[197]

Many stoichiometric ratios or antioxidant capacities for naturally-occurring catechols have been determined. Several of these catechols demonstrate impressive antioxidant capacities (quercetin (2.6), (-)-epigallocatechin (2.2), catechin (1.7) and methylgallate (2.3) calculated from the oxidation of cumene in methyl linoleate[227]).

In spite of their lower apparent antioxidant capacity, SECO **2** and SDG **1** may however have an advantage to the catechols. Flavonoid catechols are susceptible to oxidation to quinones, especially ortho-quinones, which can increase oxidative stress or react with biological molecules.[165] SECO **2** and SDG **1** cannot form ortho-quinones. In conclusion, SECO **2** and SDG **1** demonstrated that they are chain-breaking antioxidants because they trap AP radicals and do not propagate the chain of oxidation reaction.

5.2.2. Rancidity test

The Rancimat method is one technique that can be used to measure rancidity of fats and this data can be used to estimate the effect an antioxidant may have on shelf life of a food product. This technique has the advantage that it measures volatile lipid peroxidation products and that it is automated and calculates induction time, a measure of antioxidant capacity, through a curve-fitting program. The goal of these experiments was to determine whether the lignans in flaxseed would act as preservatives of an oil-based food (canola oil) and which form of lignan, SECO **2**, SDG **1** or SDG polymer **3**, would be the most effective in this system. Since BHT **17** was used as a standard in the stoichiometric ratio and is used as a commercial food preservative it was decided that the use of BHT **17** in the rancidity study may allow comparison with the results from the stoichiometric study. In addition, BHT was used instead of BHA because BHT is more heat resistant than BHA which makes it a better model compound for this study design.[150 230]

One goal of this study was to utilize conditions similar to those seen in real food systems.[149,230] All oil samples were stored in the dark at room temperature as hydroperoxide decomposition is more rapid at higher storage temperatures (>60°C) and the kinetics of oxidation are not the same as would be observed at lower temperatures.[225] An initial pilot study was performed using equivalent weights of BHT **17** and SECO **2** per 100g oil to determine a sensitivity range for this method. The concentrations used were chosen based on a typical BHT **17** level used in food preservation of 200 ppm.[272] The concentrations of 10, 20 and 30 mg/ 100 g oil correspond to 100, 200 and 300 ppm BHT **17**, respectively. In addition, the pilot study was carried out at two temperatures, 110ºC and 120ºC. These temperatures are commonly used to model frying temperature. The results for the pilot study at both temperatures show that induction time decreases over time and both antioxidants minimize the decrease in induction time in a concentration-dependent manner (Table 4.3). The results at 120ºC show that the rate of decrease in induction time is approximately double that at 110ºC over time, as would be predicted by the Arrhenius equation for every 10ºC change.[280] It was decided to use 110ºC for the remainder of the study as the results at this temperature showed greater sensitivity to changes in concentration (Table 4.4.).

Although BHT consistently demonstrates a greater protective effect than the lignans, the values were similar. Since the molecular weight of SECO **2** is 1.6 times greater than the molecular weight of BHT **17**, this implies that on a per molecule basis SECO **17** may be as potent an antioxidant as BHT **17**. To test this observation,

the Rancimat study was repeated using mole per 100g oil to compare the antioxidant ability on a per molecule basis. Two additional compounds were tested, SDG **1** and SDG polymer **3**. Kamal-Eldin *et al.* (2001) reported that the SDG polymer **3** (MW=4000) consists of a straight-chain structure composed of five SDG **1** residues interconnected by four HMGA residues.[29] It is important to note that other phenolics, including cinnamates, are present in the SDG polymer **3** and that these may contribute to the antioxidant effects.[29] However, many of these cinnamates are present as -O- glucosides which limit their potential to act as antioxidants in food systems but not in mammalian systems (personal communication with Dr. Muir). A specific wt% can not be assigned to the polymer, although a lower limit of 35 wt% SDG **1** in the polymer[30] allows an estimate of SDG **1** equivalents such that 10 mg, 20 mg, or 30 mg/100 g oil correspond to SDG **1** concentrations of 0.0044 mmol, 0.0087 mmol or 0.0131 mmol/100 g oil, respectively.

The induction time experiments showed that flaxseed lignans SECO **2**, SDG **1** and SDG polymer **3** can inhibit autoxidation in a vegetable oil (canola) model and prolong the shelf-life with similar induction times to that of BHT **17**, the positive control (Table 4.4). The ability of the SDG polymer **3** to inhibit autoxidation, at least as well as SDG **1**, may represent an economic benefit, since SDG polymer **3** requires fewer processing steps and is less expensive to isolate than SECO **2** or SDG **1** (personal communication with Drs. Muir and Westcott). Thus, it may be that SDG polymer **3** is the most economically feasible form of flaxseed lignans to use in the role of a food preservative.

The results observed for the Rancimat study are in agreement with those seen for the stoichiometric ratio results, in which all of the compounds studied were antioxidants and were in the order BHT>SECO>SDG. The glucose moieties on SDG **1** are clearly responsible for the difference in activity between SECO **2** and SDG **1** as discussed in the previous section, but what remains unclear is whether this is the result of solubility or reactivity of the alcohol groups on SECO **2**.

BHT **17** and SECO **2** were dissolved directly in the bulk oil, SDG **1** and SDG polymer **3** do not dissolve in oil as well and required a co-solvent (1 mL ethanol), therefore co-solvent was used for all of the compounds tested. However, the SDG polymer **3** unexpectedly showed higher antioxidant activity than SDG **1** in this canola oil system. The SDG polymer **3** has been observed to elute after SDG **1** by reversed-phase HPLC, indicating that the SDG polymer **3** is more lipophilic than SDG **1**.[297] Esterified HMGA residues in the SDG polymer **3** appear to increase the lipophilicity of the polymer compared to SDG **1**.[29,30,298] The observed antioxidant activity of the SDG polymer **3** confirms that glucosylated lignans can maintain antioxidant activity, which may be enhanced with increased lipophilicity. What the contribution of the HMGA substituent and the trace phenolics present in the polymer to antioxidant capacity is unknown and beyond the scope of this study. Variability in the SDG **1** content in the polymer is also a potential problem with the assessment of the polymer as a food preservative. In this study, all of the SDG polymer **3** used was derived from a single batch, however without a standardized protocol for

polymer isolation, it will be necessary to determine % SDG **1** content accurately for any future comparative studies.

What is also unknown is why SECO **2** does not protect canola oil as well as BHT **17**. This may be the result of differences in lipid solubility, or that the mechanism of antioxidant activity of SECO **2** differs from that for BHT **17**, an idea that will be explored further in the discussion of the product study results.

Another method for expressing results from the Rancimat study is to use a protection factor (PF) which is a ratio of IT for each sample versus IT for the blank. [150,225,299] The *n*-values and the PF values (Table 4.5) at 120 days for the antioxidants tested allows for a comparison of the results for these two systems. The PF for BHT **17** is lower than the *n*-value, which may indicate that the Rancimat method is less sensitive to minor differences in antioxidant capacity. This comparison illustrates that a similar trend exists for the two systems, although it remains difficult to treat these numbers as quantitative.

For the purpose of cooking and frying oils there are four factors that good antioxidants must possess: lipid solubility, heat stability, the presence of active hydroxyl groups and the ability to maintain clarity (ie. the compound does not cause cloudiness).[149] SECO **2** and SDG polymer **3** meet the criteria of lipid solubility and active hydroxyl groups, their heat stability and ability to maintain clarity would require further investigation. The results reported in this study can only be used to infer the potential use of flaxseed lignans as food preservatives in oil based food systems. The use of the lignans in other food systems such as oil in water emulsions

would require experiments carried out in the appropriate systems. It would also be interesting to determine whether a synergistic antioxidant effect is observed for SECO **2** and SDG **1** in combination, for which an emulsion may be an appropriate model system. Studies such as these would also require a clear understanding of the solubility, stability and partitioning of SECO **2**/SDG **1** in oil in water or water in oil emulsions.

In conclusion, the two oxidation model systems demonstrated the potential for SECO **2**, SDG **1** and SDG polymer **3** to act as antioxidants in oil-based foods. First, the stoichiometric ratio in a liposomal system established that this was a satisfactory assay to measure the ability of SECO **2** and SDG **1** to inhibit APPH **47**-catalyzed lipid peroxidation.[227] The stoichiometric ratio for SECO **2** and SDG **1** were 1.5 and 1.1-1.2, respectively, compared to BHT **17** (2.0), indicating that both SECO **2** and SDG **1** delayed AAPH **47**-induced lipid autoxidation likely through a combination of peroxyl radical chain termination reactions and by scavenging AP radicals. These results demonstrated that SECO **2** and SDG **1** are less potent antioxidants compared to BHT **17**, however they have potential advantages over BHT **17** in that they are natural antioxidants that have potential health benefits associated with their consumption.[129,153,277] It should be noted however, that before SECO **2** or SDG **1** could be used in this capacity, more in-depth toxicological studies are warranted to ensure that prolonged consumption of flaxseed lignans is safe.

Second, it was determined that the Rancimat was an appropriate analytical tool for the measurement of inhibition of autoxidation mediated by flaxseed lignans in comparison with BHT **17**-mediated inhibition of autoxidation. Both model systems showed that as an antioxidant, SECO **2** provides better protection than SDG **1**. The observed induction times were consistent with the calculated stoichiometric ratio (Table 4.5), and illustrative of two appropriate oxidation models.

The activities of antioxidants depend not only on their structural features (e.g. activities towards peroxyl and other active species), but also on many other factors, such as concentration, temperature, light, type of substrate, physical state of the system, as well as on microcomponents acting as pro-oxidants or synergists.[225] Several important factors that may affect the results of antioxidant studies in lipid systems are the initial concentration of primary autoxidation products, such as hydroperoxides in the lipid and free fatty acids. Primary oxidation products may reduce both the efficiency of the antioxidants and the time for radical-mediated consumption of antioxidant.[149] Free fatty acids may demonstrate a pro-oxidant effect and should be minimized. Other lipid microcomponents, such as metals, fatty alcohols and mono- and diacylglycerols, have been also shown to decrease the effectiveness of phenolic antioxidants.[149] In the present study, attempts were made to minimize the contribution of these factors. DLPC was stored appropriately to keep hydroperoxide concentrations low. Lipids were chosen that should have little free fatty acid composition and they were free of endogenous antioxidants. High purity water was used to minimize the presence of metals such as Fe and Cu. It

should be noted however, no attempts were made to determine the background levels of these components.

5.3. SECO 2 antioxidant reaction products from AAPH 47-mediated oxidation

A pilot study was carried out to optimize the reaction conditions for AAPH **47**-catalyzed oxidation of SECO **2.** Three different concentrations of AAPH **47** (0.05, 0.1, 0.2 M) based on literature methods,[300-302] were tested and 0.1 M AAPH **47** was chosen as this concentration produced sufficient levels of reaction products for analysis. A time course study (up to 72 h) was performed and it was determined that >90% of SECO **2** had been consumed by 5 h and sufficient amounts of the major oxidation products were observed to allow for isolation and purification. In addition, the oxidation reaction was carried out at two different temperatures (50ºC and 60ºC) and 60ºC was chosen as optimal. AAPH **47** undergoes thermal decomposition in solution with the loss of N_2 to produce two carbon centred radicals (AP). The AP radical can add O_2 to form a peroxyl radical, although carbon centred radicals often predominate. There are two notable limitations to the product study, AAPH **47** tends to form carbon-centred radicals as opposed to peroxyl radicals and lipid is absent from the reaction system. AAPH **47** was used due to it's favourable solubility properties that allowed this study to be carried out for both SECO **2** and SDG **1** and because unlike AMVN, the products of thermal decomposition do not possess a chiral centre. Although lipid was absent in the product studies to simplify

isolation and identification of the potential oxidation products, this model system still provides valuable information about the possible antioxidant reactions of SECO **2**.

Oxidation of SECO **2** with AAPH **47** indicated that compounds **48** and **52** were formed rapidly and decomposed after 90 min, whereas compounds **49, 50** and **51** formed later (section 4.2, Figure 4.6). It would be expected that the phenolic OH would preferentially undergo H-atom abstraction.[200] The results reported here, however, indicate that the early-forming compounds involved the aliphatic alcohols of SECO **2** in the oxidation reaction. This is surprising given that the O-H bond energy of an aliphatic alcohol (104.5 kcal mol^{-1})[303] is greater than for a phenolic O-H (72.6 kcal mol^{-1}).[225] Several possible reaction pathways leading to the formation of **48** and **52** can be envisioned. One possibility is that the aliphatic oxygen-centred radical **2a** forms directly from the AP radical mediated H-atom abstraction from the SECO **2** aliphatic OH. A hydrogen-bonding interaction between the aliphatic alcohols may lower the pKa of the alcohol, which could produce alkoxide ion in low amounts which would be more susceptible to radical formation.[304] Alternatively, an intramolecular H transfer from the aliphatic alcohol to the initially formed phenoxl radical **2c** could occur (Figure 5.1). A third, albeit less likely, possibility cannot be ruled out in which an intermolecular H transfer from the aliphatic alcohol of one molecule of SECO **2** to the phenoxyl radical **2c** of another molecule of SECO **2** occurs. The products derived from the alkoxyl radical **2a** were unexpected and appear to be unique. It would be beneficial to obtain crystal

structures of these products as further confirmation of this unexpected pathway, however this will require the preparation of further amounts of compounds **48**, **51** and **52** via AAPH **47** catalyzed oxidation of SECO **2**.

The peroxide structure proposed for **52** was probed further by determining the amount of peroxide present using a peroxide-value test.[283] This method is sensitive to alkyl peroxides and can be used for quantitation, although the results in this study were near the lower region of the calibration curve. The results indicate the presence of 1.8 peroxide equivalents per one molecule of **52**, close to the expected peroxide number of 2.0 per molecule of compound **52**. Previous studies have also showed the formation of peroxides from phenolic compounds with methyl or hydroxyl substitution.[305]

The intermediate alkoxyl radical **2a** is proposed to be involved in the generation of the early-forming oxidation compounds (**48** and **52**) while the phenoxyl radical **2c** would lead to the late-forming oxidation compounds (**49**, **50** and **51**). It would be anticipated that the phenoxyl radical **2c** is more stable than the alkoxyl radical **2a**. The results presented here are in agreement with this since the majority of the products are derived from the phenoxyl radical pathway and products from the alkoxyl radical **2a** appear to form more rapidly and/or be converted to secondary products faster.

A reaction pathway can be proposed which fits the experimental data reasonably well. After formation of intermediate **2a**, rapid dimerization to form compound **48** would occur (Figure 5.2). Since **48** was shown to be transient in the

time-course and AAPH mediated oxidation of **48** lead to the production of **52**, **51** and SECO **2**, this suggests that dimerization of **2a** to form an aliphatic peroxyl linkage is reversible. The alkoxyl radical **2a** from this reversible reaction could capture a hydrogen atom from solution to produce SECO **2**, which may also explain the greater overall yield of phenoxyl radical derived products. Compound **52** could be formed from further oxidation of **2a**, in which subsequent formation of a second alkoxyl radical (not shown) is followed by oxidation. The source of the additional oxygen atoms in **52** could be either from solvent (H_2O) or atmosphere (O_2). Mass difference experiments using either $H_2{}^{18}O$ or ${}^{18}O_2$ may be useful in the future for determining the origin of these incorporated oxygen atoms. The stable product, **51**, appears to result from formation of **52b**, the phenoxyl radical of **52** (Figure 5.3). The time course for the oxidation of **48** shows loss of **52** coupled to the appearance of **51**, which strongly suggests that addition of the AP radical must happen after oxidation of SECO **2** to **52** and not as a result of initial formation of **50** and subsequent oxidation of the aliphatic alcohols. This agrees with the observations that the aliphatic alcohol radicals seem to form and react faster than the phenoxyl radicals.

5.1a*

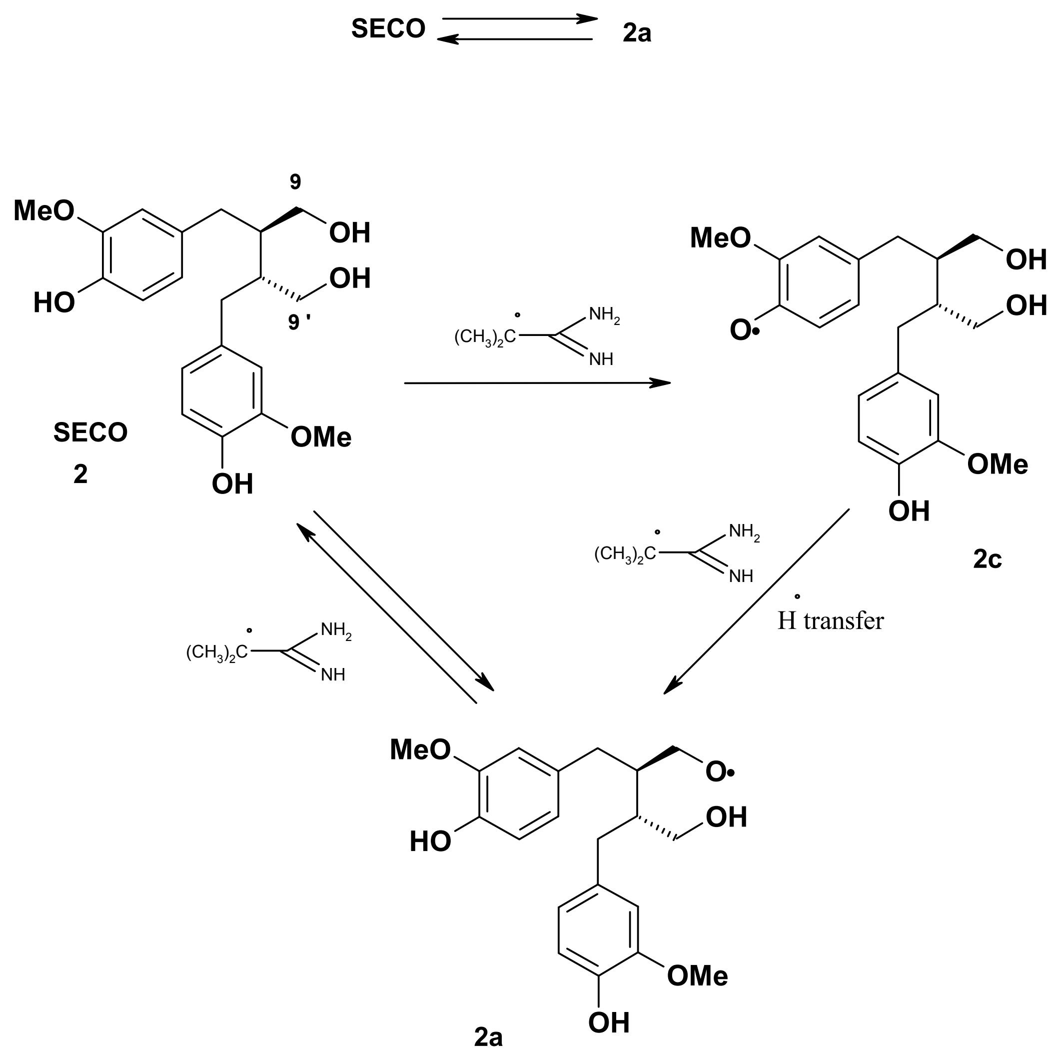


Figure 5.1. Proposed reaction scheme for the formation of **2a**.
*For simplicity, Figure 5.1a shows the reactions by their product numbers.

5.2a*

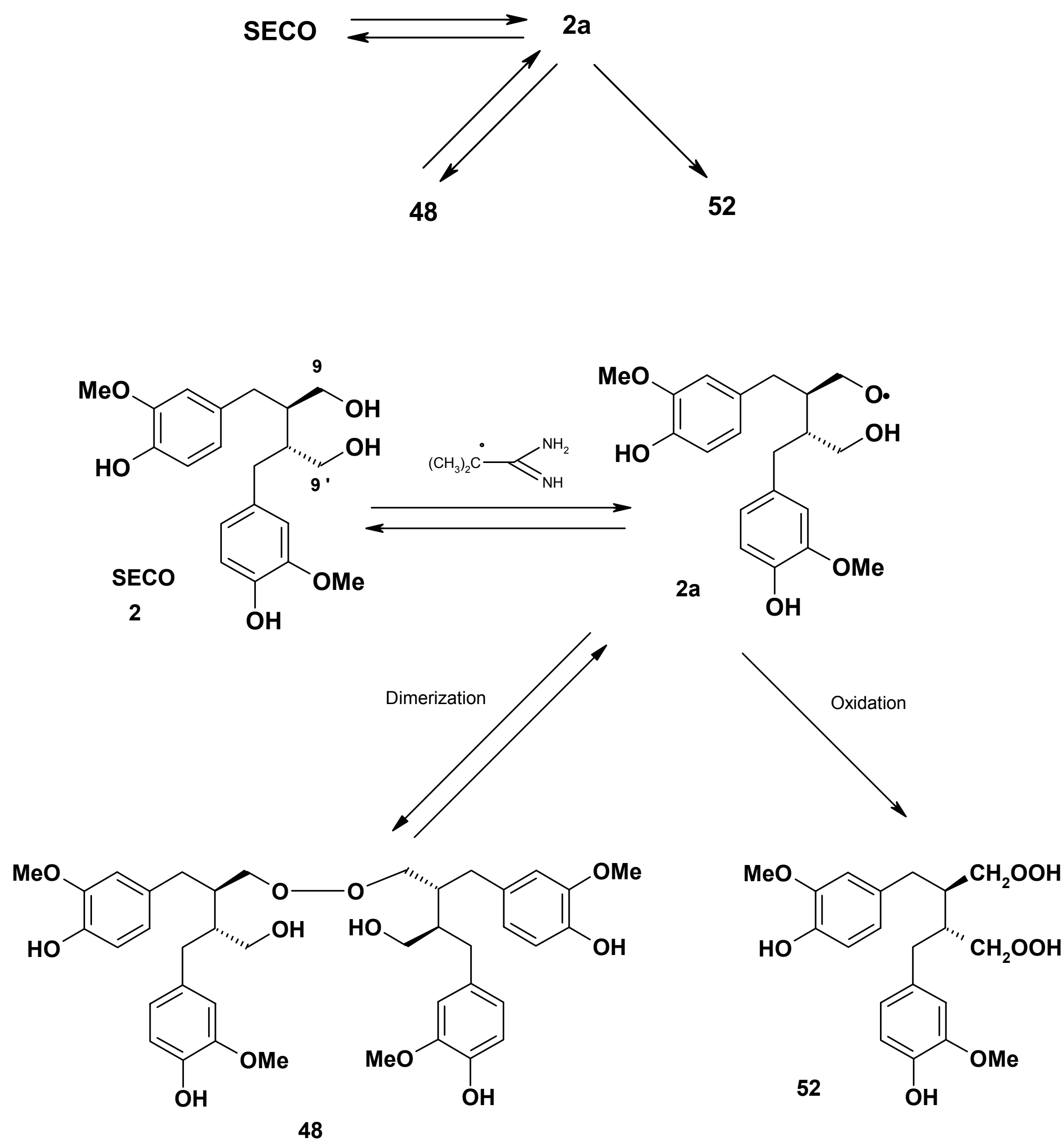


Figure 5.2. Proposed reaction scheme for the formation of compounds **48** and **52** from SECO **2** via **2a**.
*For simplicity, Figure 5.2a shows the reactions by their product numbers.

Compound **50** was determined to be the major product of AAPH **47** mediated SECO **2** oxidation. Compound **50** would be derived from the anticipated reaction pathway, AP mediated H-atom abstraction from the phenol to yield the resonance stabilized phenoxyl radical **2c** (Figure 5.4). The phenoxyl radical **2c** would then undergo a radical recombination with an AP radical to form the stable product **50** (Figure 5.4). This reaction is indicative of a chain-breaking antioxidant and suggests that SECO **2** likely functions in this way. Compound **49**, a dimer of SECO **2**, is also derived from the initially formed phenoxyl radical **2c**. Unlike the formation of compound **48** however, dimerization does not occur via an O-O peroxyl linkage, instead a carbon-carbon bond is formed between the phenyl rings. The phenoxyl radical is resonance stabilized and one of the resonance contributors would place the unpaired electron on the unsubstituted carbon ortho to the phenol OH (Figure 5.5). Thus, formation of **49** can be rationalized as a dimerization between two of the carbon-centred resonance contributors shown in figure 5.5. The initial product of dimerization would be expected to have a di-keto structure. The two aromatic rings involved in the dimerization however are not present in the keto-form, indicating that aromatization drives the equilibrium from the keto to the enol (phenol) form. It is possible that dimerization of **2c** to **49** is reversible as is suggested by the time-course data, however due to the limited amount of product isolated, an AAPH **47** mediated oxidation of **49** could not be performed to confirm this.

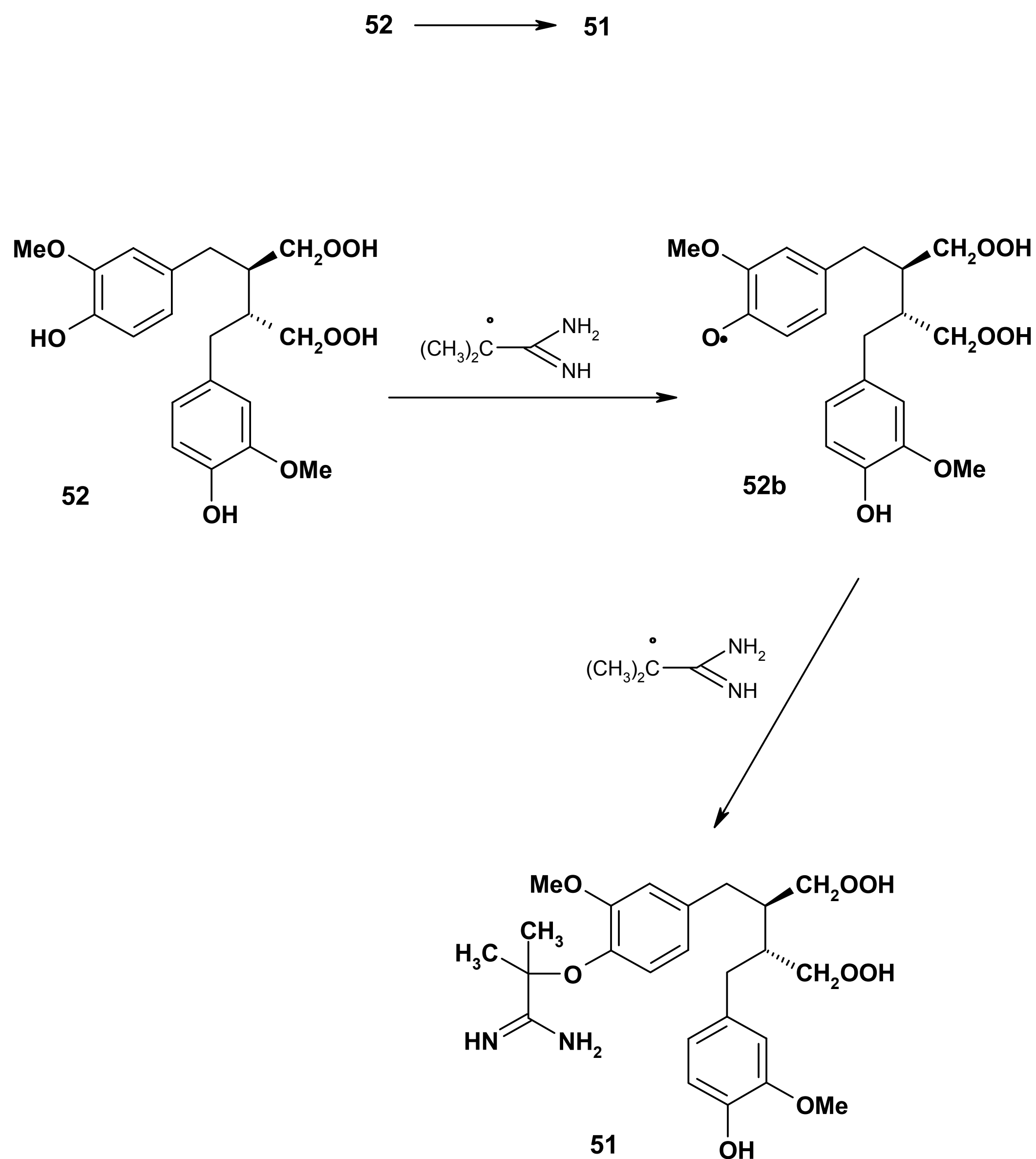


Figure 5.3. Proposed reaction scheme for the formation of compound **51**.
*For simplicity, Figure 5.3a shows the reactions by their product numbers.

5.4a*

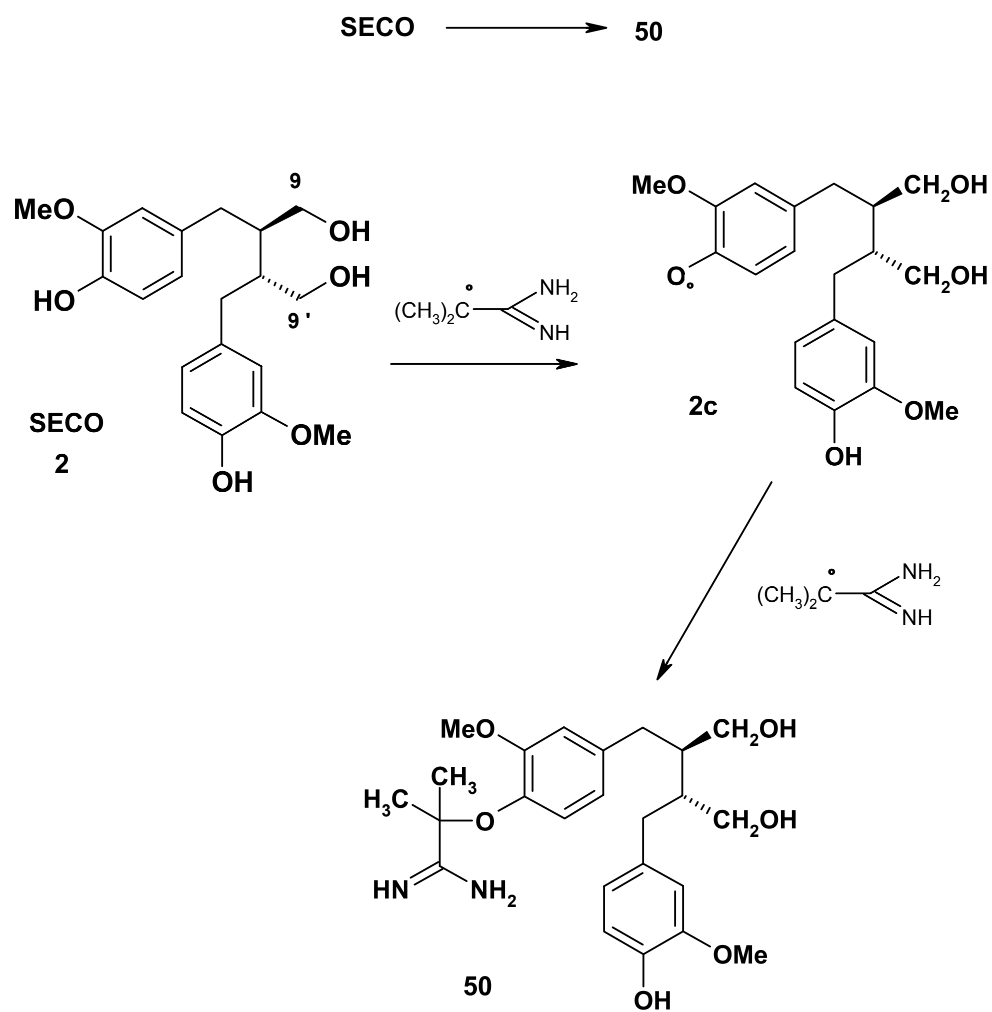


Figure 5.4. Proposed reaction scheme for the formation of compound **50**.
*For simplicity, Figure 5.4a shows the reactions by their product numbers.

5.5a*

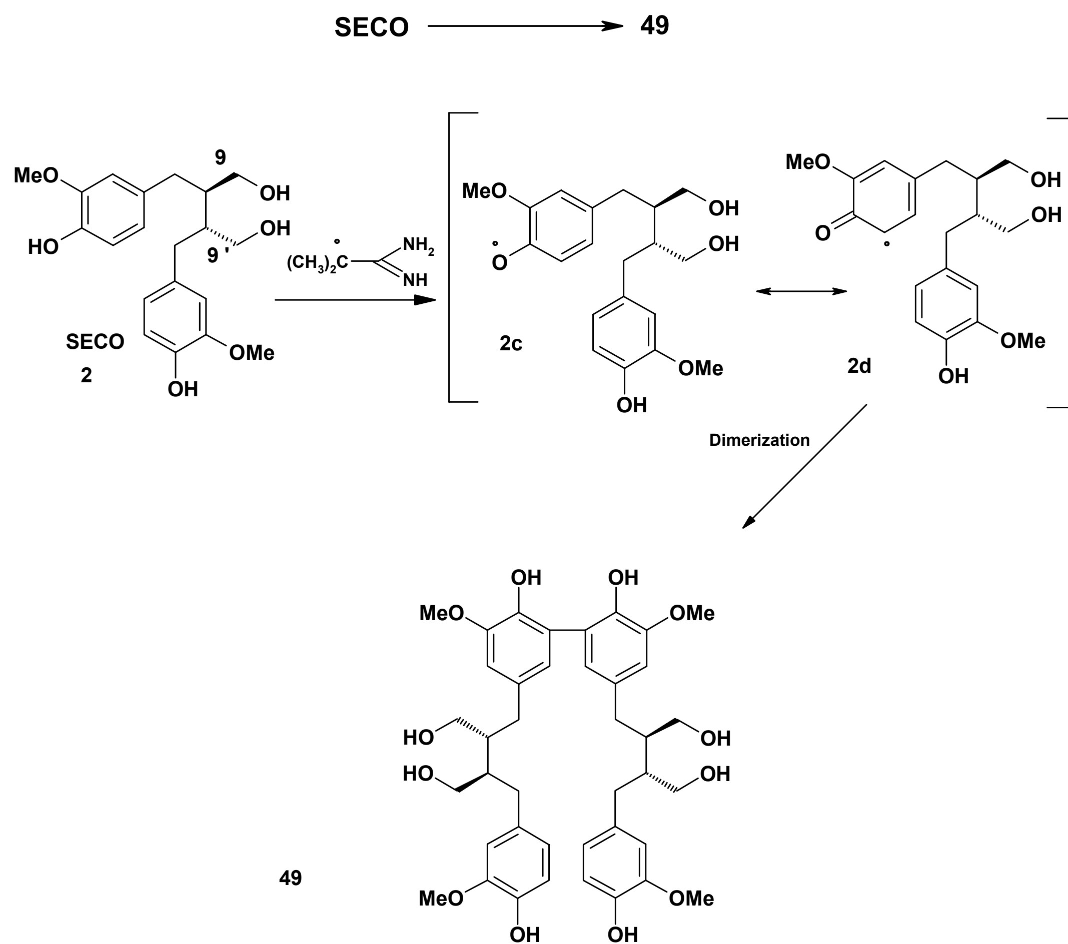


Figure 5.5. Proposed reaction scheme for the formation of compound **49**.
*****For simplicity, Figure 5.5a shows the reactions by their product numbers.

The data from the oxidation experiments reported here supports the proposed reaction mechanism of SECO **2** as a scavenging agent that breaks chain of oxidation (Figures 5.6 and 5.7). In the proposed oxidation pathways, compound **48** is formed from dimerization of **2a** that subsequently is converted to **52** and then to **51** (Figure 5.6). In addition, the final compounds **50** and **51** can trap the carbon-centered AP radicals. Compound **52** also is an intermediate compound of oxidation, which is converted to compound **51** with the ability to scavenge the carbon-centered AP radical. It appears that under these conditions, compounds **48** and **52** undergo further oxidation to yield compound **51**.

Recently, a similar study was published which examined the AAPH **47** (referred to as ABAP) mediated oxidation products of SECO **2**.[152] In that paper, all of the products reported in this thesis were not observed. Only products from the phenoxyl radical pathway (**49** and **50**) were observed, in addition to two other products, a dimethyl furanone of SECO **2** and lariciresinol. The authors suggest that the dimethyl furanone is formed from further reaction of compound **50** and that lariciresinol is formed via a quinone methide intermediate in which an intramolecular cyclization occurs between an alcohol group and the terminal CH group of the quinone methide. In this paper, NMR data was presented only for the dimethyl furanone, the only supporting data provided for the other compounds was from LCMS. Therefore, this study is the first to accurately determine the structures of the AAPH **47** mediated SECO **2** metabolites.

It is important to note that although the work reported in this thesis did not observe lariciresinol, it is possible that small amounts may have been formed. Lariciresinol is difficult to separate from SECO **2** using reverse phase HPLC and it may have co-eluted under the conditions used or been one of several minor products that were not characterized due to insufficient product amounts. Since this thesis reports SECO **2** oxidation products resulting from an alkoxyl radical **2a**, it is possible that lariciresinol in Eklund *et al.,*[152] study was formed through a similar alkoxyl radical, although this cannot be proven with the present data.

There were three major differences in Eklund *et al*.,[152] experimental protocol from that used in this thesis which would be expected to influence the reaction products observed.[152] Eklund *et al*., used a higher temperature (70ºC), a longer incubation time (72 h versus 5 h) and an AAPH **47**:SECO **2** ratio of 3:1 versus 10:1 used in this study. It was also not clear what their final concentrations of AAPH **47** and SECO **2** were. If Eklund *et al*., used lower concentrations than those used in this study, that could explain the need for higher reaction temperatures and longer reaction times.

5.6a*

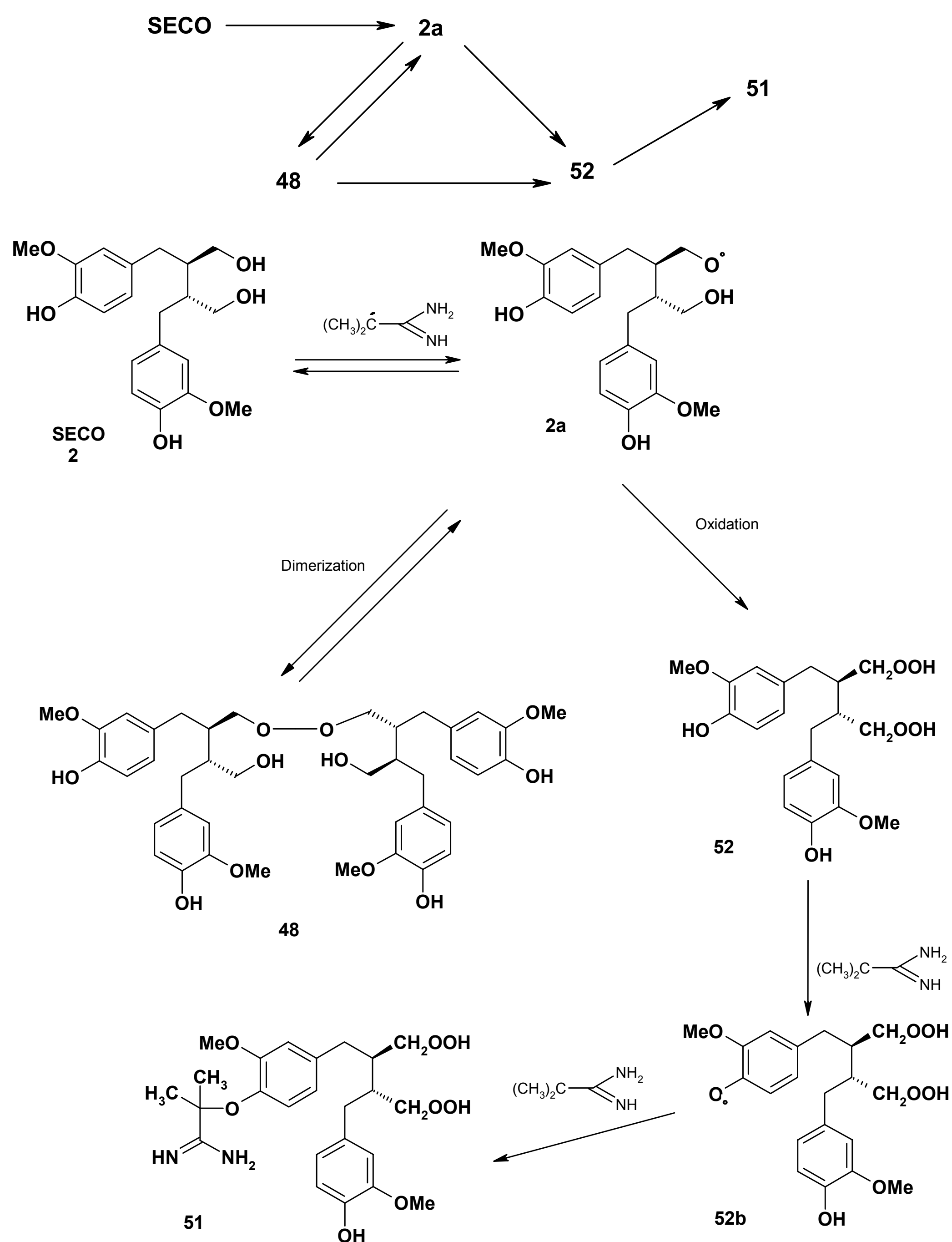


Figure 5.6. Proposed reaction scheme for SECO **2**:AAPH **47** oxidation from hydroxyl groups on C-9 positions.

*For simplicity, Figure 5.6a shows the reactions by their product numbers.

5.7a*

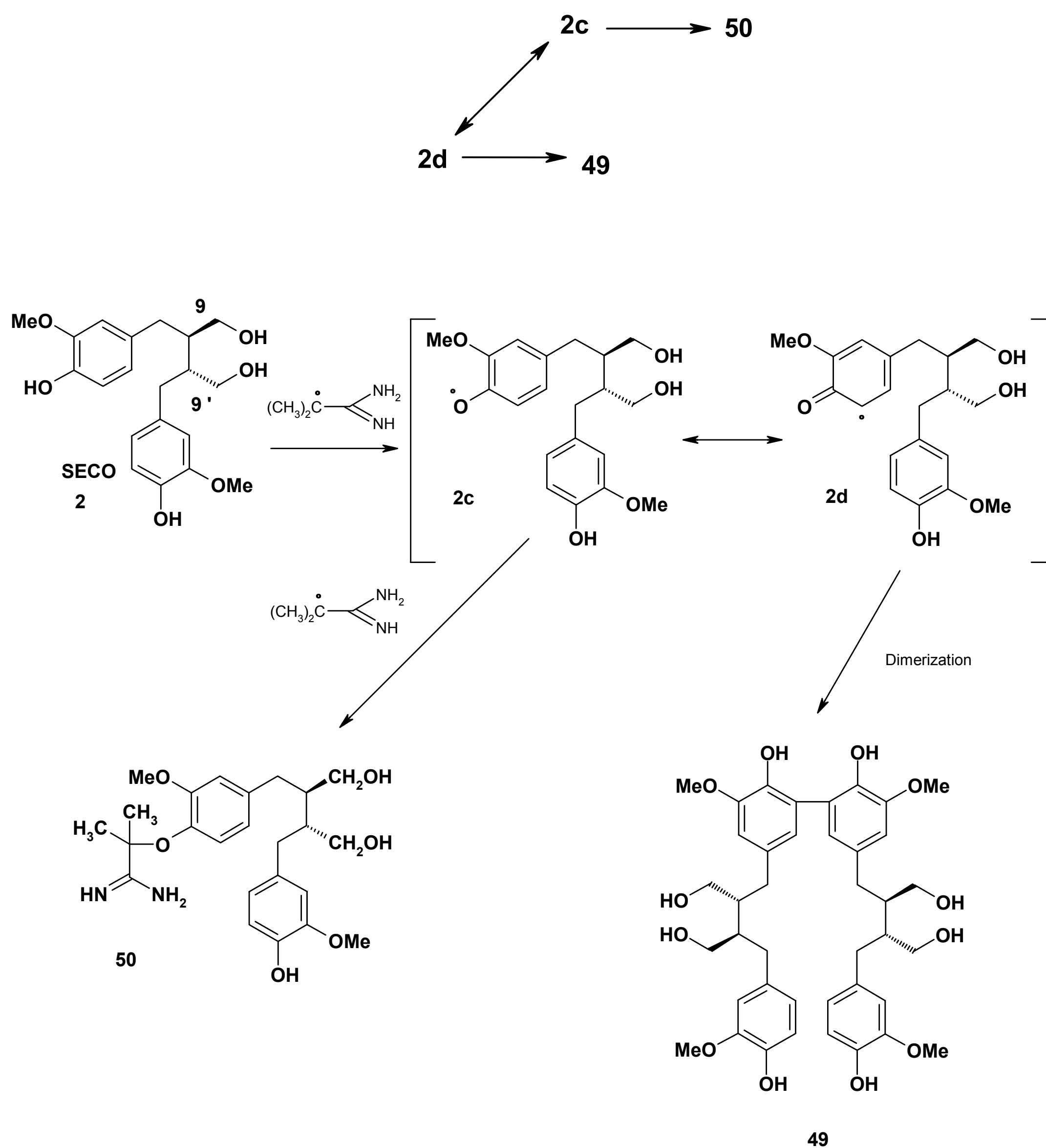


Figure 5.7. Proposed reaction scheme for SECO **2**:AAPH **47** oxidation from aromatic hydroxyl groups.

*For simplicity, Figure 5.7a shows the reactions by their product numbers.

The dimethyl furanone observed by Eklund *et al*., would not be expected under the less drastic conditions used in this study as they were apparently the result of decomposition of **50**. The excess AAPH **47** used in this thesis likely influences the formation of the alkoxyl radical-mediated species. Given the apparent instability of the alkoxyl radical derived products (**48**, **51** and **52**) the higher temperature and extended reaction time would likely lead to decomposition of these products which may explain why Eklund *et al*., reported a greater loss of material to polymeric products than observed in this thesis.

There are several antioxidant reaction pathways that would be predicted for SECO **2**, using BHT **17** as a model of phenol antioxidant reactions. The most likely reaction pathways would involve initial loss of a phenolic hydrogen atom to form a resonance stabilized phenoxyl radical (**2c**) followed by radical recombination with AP or another radical species at either the phenoxyl oxygen, the C-5 carbon or the C-1 carbon. Ideally, this would result in the removal of two radicals per phenol ring, potentially leading to a total of four radicals consumed per molecule of SECO **2**. The stoichiometry study indicates that SECO **2** consumes substantially less than four radicals per molecule (1.5). There are several possible explanations for this result including limited incorporation of SECO **2** into the liposomes and competing reactions that would generate radicals or have a net radical consumption of zero. The reaction pathway outlined in figure 5.7 demonstrates that formation of an AP-SECO **2** product, compound **50**, does occur and would have a stoichiometric value of two associated with it. Formation of compound **49** would however have a net

stoichiometric value of one, since each molecule of SECO **2** donates a hydrogen atom to consume one radical and form **2c** but the dimerization only consumes molecules of **2c** and not other radical species. There is no evidence that oxidation occurs on both aromatic rings of SECO **2**, as can be seen in both product **49** and **50**. This is interesting since an intramolecular dimerization of **48** or **49** should proceed rapidly, provided the phenoxyl or alkoxyl radicals can be formed. This suggests that only one phenol in SECO **2** is involved in antioxidant reactions, indicating that a radical scavenging model based on the number of aromatic hydroxyl groups may not apply to this group of compounds. This observation agrees with a report by Metzler in which the radical scavenging ability of SECO **2**, as measured using the Ferric Reducing/Antioxidant Power assay, was comparable to that of a monophenolic analogue, 2-methoxy-p-cresol.[306] Further antioxidant studies on selected analogues would be necessary to more fully understand this phenomena.

The products derived from the reaction pathway outlined in figure 5.6 would be expected to provide a smaller contribution than those from the reaction pathway in figure 5.7 as they are formed in smaller amounts. Formation of the proposed initial alkoxyl radical would result in the consumption of one radical. It would initially appear that dimerization to form **48** would result in a net stoichiometric value of one (see **49** above), however since **48** decomposes over time, this contribution must be considered in further reactions that occur. The contribution to antioxidant capacity from **51** and **52** is more difficult to rationalize. Compound **52** is proposed to form via the alkoxyl radical **2a** and subsequent oxidation. Oxidation of

the second aliphatic alcohol would provide an additional stoichiometric value of one, however subsequent oxidation to the peroxyl species would likely involve the generation of a radical species therefore negating the radical scavenging effect described earlier. Compound **51** would be anticipated to have a stoichiometric value comparable to **50** as a result of the AP-phenoxyl product.

Since the different oxidative metabolites form at different rates, it is difficult to try and add their contributions to arrive at a stoichiometric ratio. What can be stated with some certainty is that two oxidation products (**50** and **51**) should contribute a stoichiometric value of 2, the remaining oxidation products should contribute a stoichiometric value of 1 (**48**, **49** and **52**).

Another point that must be considered is the potential reactivity of the alkoxyl radical. A lipid alkoxyl radical is 10^4-10^6 times more reactive towards hydrogen atom abstraction than a lipid peroxyl radical, suggesting alkoxyl radical formation may be pro-oxidant in nature.[307] In this case, **2a** would abstract a hydrogen atom from a lipid molecule, starting a new chain reaction and contributing further to radical propagation. It may be that oxidation products from **2a** cause an increase in lipid peroxidation and reduce the antioxidant effect observed from the phenoxyl radical pathway. The antioxidant reactions for **2a** may prevent this pro-oxidant effect, provided their rate of formation is at least comparable to $L^\bullet$ formation. It is not unreasonable to speculate that the antioxidant stoichiometry of SECO **2** could be higher, but is limited by the competing pro-oxidant reactions of SECO **2**.

The aromatic hydroxyl groups at the C-4 position and the aliphatic hydroxyl groups at the C-9 and C9' positions appear to be the preferred oxidation sites for AAPH **47**. The contribution of hydroxyl groups in the oxidation of SECO **2** by AAPH **47** is in keeping with the previously reported importance of hydroxyl groups in radical scavenging and the antioxidative potential of phenolic compounds.[302,308] The formation of dimers like **48** and **49** has been reported previously as products of oxidation for other phenolics including (-)-epicatechin in green tea[242,266,267] and vitamin E. [309-311]

Compounds **48** and **49** had extinction coefficients twice that of SECO **2**, which would be expected since they are dimers and contain four phenol rings in their structures compared with SECO **2** (two phenol rings). The extinction coefficients for compounds **50** and **51** were slightly higher than SECO **2**, as a result of the AP-substituent, since products from thermal decomposition of AAPH **47** had a low absorbance at 280 nm with an extinction coefficient (L mol^{-1} cm^{-1}) of 586. It was interesting that compound **51** showed a maximum UV absorption at 258 nm, which was lower than the UV absorbance for other compounds, and has been previously reported for 3,4-dialkoxy substituted lignans.[312]

Oxidation compounds of SECO **2** with AAPH **47** showed they were scavengers of carbon-centred AP radicals and as a result, may act as antioxidants. Previous investigations have shown that flaxseed lignans have antioxidant properties.[102,151,288,313,314] This study has shown: a time-course for the reaction of SECO **2** with AAPH **47**, identified the major products derived from the phenoxyl

radical **2c** by NMR and LCMS and proposed a reaction pathway for their formation, and for the first time identified the major products derived from the SECO **2** alkoxyl radical **2a** by NMR and LCMS and proposed a reaction pathway for their formation.

In conclusion, the oxidation of AAPH **47** to produce carbon-centred AP radicals upon thermal decomposition provides a good model to study the antioxidant reactions of SECO **2** *in vitro*. The antioxidant mechanisms of SECO **2** would be based on the ability of SECO **2** to donate a hydrogen atom and trap free radicals, consequently inhibiting oxidative chain reactions. SECO **2** forms non-radical compounds, which do not easily participate in other radical reactions, several of which (e.g. **50** and **51)** are similar to products observed with other chain breaking antioxidants such as BHT **17** and vitamin E.[197,215,228] Compounds **50** and **51** are non-radical compounds that result from scavenging of an AP radical, which supports SECO **2** having a role as a chain-breaking antioxidant *in vitro*. The formation of alkoxyl radical mediated products was unexpected and it is unknown at this time whether the reactions associated with the alkoxyl radical would be antioxidant or pro-oxidant in nature.

5.4. SDG 1- antioxidant reaction products from AAPH 47-mediated oxidation

AAPH **47** was also used for the generation of AP radicals to oxidize SDG **1**. The same initial conditions used for the oxidation of SECO **2** were used for SDG **1**, except that the incubation time was shortened to 4h. The results of the SDG **1**

oxidation indicated that products **55** and **58** are formed early and decompose after 50 minutes, product **57** forms early and continues to increase throughout the incubation and product **56** forms later in the reaction (section 4.6, Figure 4.11). An AAPH **47** control reaction helped to distinguish the products of thermal decomposition of AAPH **47** from the major SDG **1** oxidation compounds. The identification of SDG **1** oxidation compounds in this thesis provides the first unambiguous proof that the principal site of antioxidant reactions on SDG **1** is the phenol hydroxyl group at position 4/4′. In addition, there was no evidence of any oxidation reactions occurring on the glucose groups.

A reaction pathway can be proposed which fits the experimental data of the AAPH **47**-mediated SDG **1** oxidation. The first step in the formation of all of the isolated SDG **1** oxidation products would be abstraction of a hydrogen atom from the phenol by AAPH **47** to yield the intermediate phenoxyl radical **1a** (Figure 5.8). This radical would be resonance stabilized and would be anticipated to react at several sites including the phenoxyl oxygen (via **1a**, figure 5.8), C-5 (via **1b**, figure 5.9) and C-1 (via **1c**, figure 5.11). Intermediate radical **1a** would lead directly to the generation of the stable, late forming oxidation compound **56** whereas resonance contributors **1b** and **1c** would lead to the formation of the early forming oxidation compounds (**55** and **58**) (Figure 5.9-5.11).

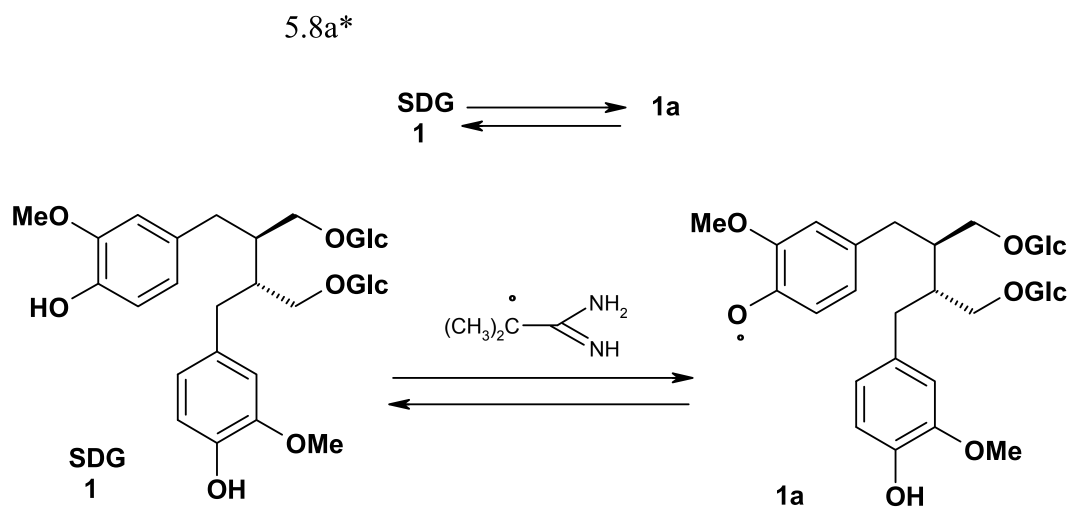


Figure 5.8. Proposed reaction scheme for the formation of **1a**.
*For simplicity, Figure 5.8a shows the reactions by their product numbers.

Compound **55** is a dimer of SDG **1** in which a carbon-carbon bond is formed and would be derived from the carbon-centred radical resonance contributor **1b** (Figure 5.9). A radical-radical recombination, similar to that proposed for the SECO **1** dimer **49**, would produce **55**. Since the SDG **1** time-course showed a decrease in compound **55** over time, a further oxidation of compound **55** with AAPH **47** was conducted. This experiment showed that AAPH **47**-mediated oxidation of compound **55** resulted in the formation of SDG **1**, **56** and **58** indicating relative instability of the carbon-carbon bond in **55** that was not observed for **49**. It may be that since **49** was a late-forming compound, compared to the early-forming **55**, significant decomposition of **49** would require a longer incubation time. The appearance of SDG **1** in the oxidation of **55** suggests that **1a** can trap a hydrogen atom under these conditions. Compound **55** also appears to isomerize from the keto to the enol (phenol) form.

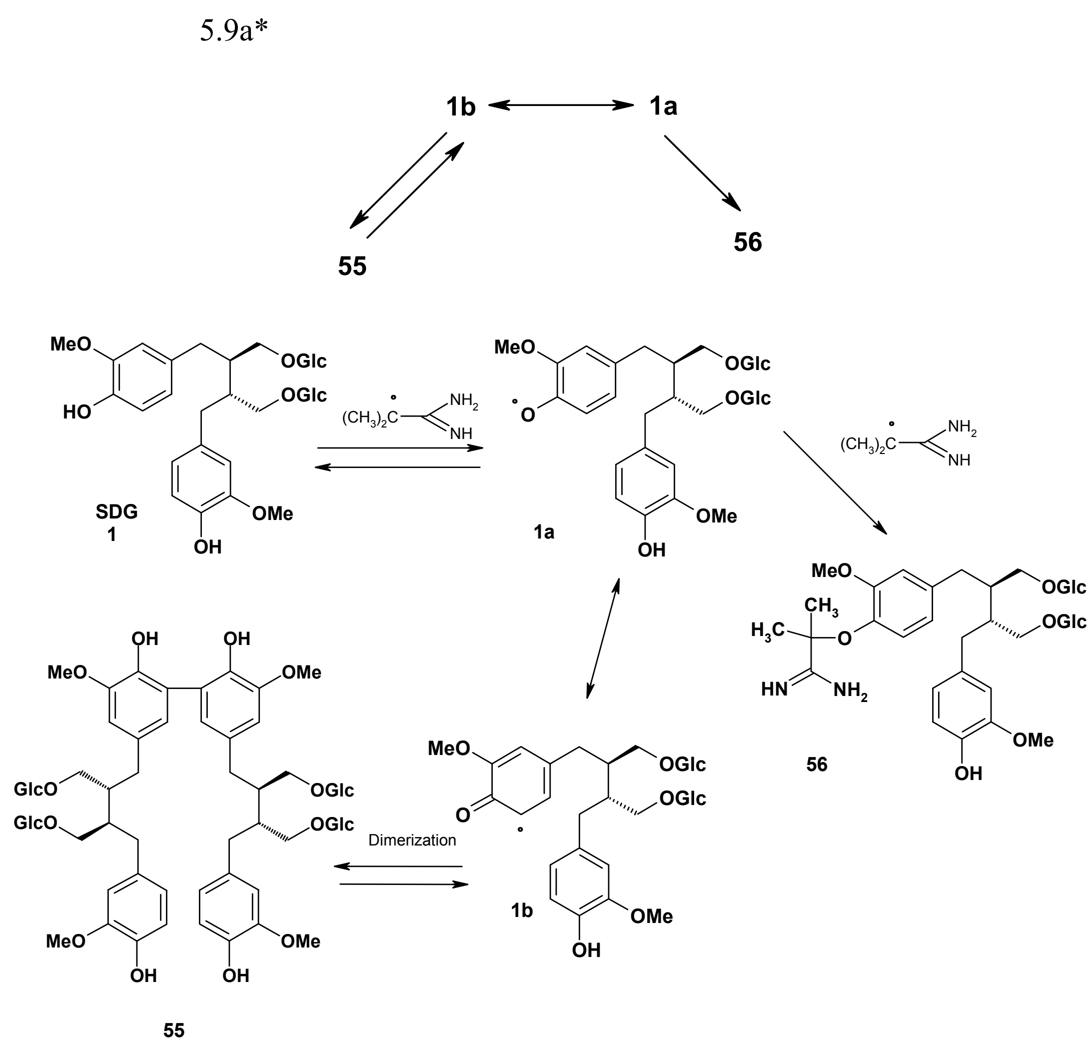
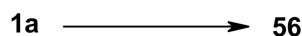


Figure 5.9. Proposed reaction scheme for the formation of compound **55**.
*****For simplicity, Figure 5.9a shows the reactions by their product numbers.

The major oxidation product found in this study was compound **56**. Compound **56** is a stable, non-radical compound formed directly via trapping of an AP radical by **1a** (Figure 5.10). This agrees with the observations for the SECO **2** oxidation in which compound **50**, the product of **2c** and an AP radical, was the major oxidation product.

5.10a*



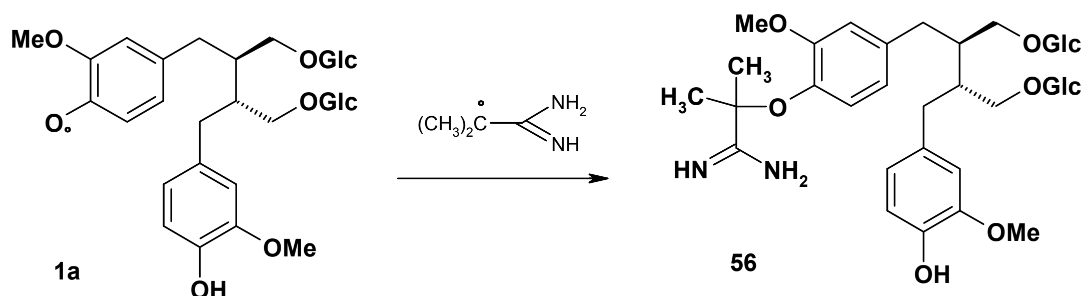


Figure 5.10. Proposed reaction scheme for the formation of compounds **56**.
*For simplicity, Figure 5.10a shows the reactions by their product numbers.

Compound **58** has a dienenone structure in one of the aromatic rings which would appear to result from the reaction of $HO^{\bullet}$ or H_2O with resonance form **1c**, the C-1 carbon-centred radical (Figure 5.11). Formation of a dieneone product is consistent with the products that would have been predicted from BHT **17** antioxidant reactions. Compound **57** is unique because it is the only SDG **1** oxidation compound in which both phenoxyl groups undergo reaction (Figure 5.12). Peroxide-value tests confirmed the presence of a peroxide in compound **57** with a total number of 1.7 peroxides per molecule of **52**. This is close to the expected peroxide number of 2.0 per molecule of compound **57** with 2 hydroxyl groups.

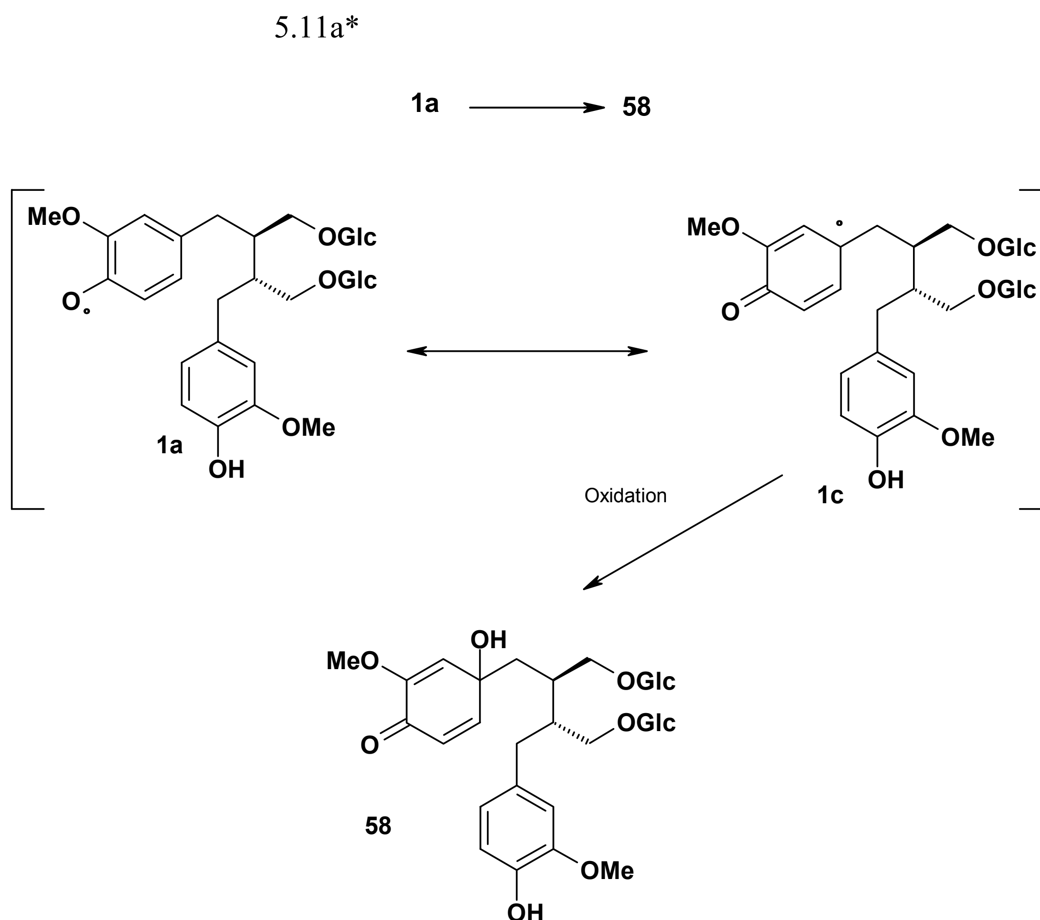


Figure 5.11. Proposed reaction scheme for the formation of compound **58**.
*For simplicity, Figure 5.11a shows the reactions by their product numbers.

Previous studies also showed the formation of peroxide from phenolic compounds with methyl or hydroxyl substitution.[305] As stated earlier for the SECO **2** oxidation products that had incorporated oxygen atoms, mass difference experiments using either $H_2^{18}O$ or $^{18}O_2$ may also be useful here for determining the origin of these incorporated oxygen atoms. The maximum UV absorption of compound **57** (269 nm) demonstrated the greatest change from SDG **1** for all of the SDG **1** oxidation products. It was interesting that the maximum UV absorption of compound **57** was

very similar to compound **51** after oxidation of SECO **2** with AAPH **47** (section 4.1, Table 4.1) and to those found previously for lignans with a 3,4 dialkoxy butyrolactone.[312]

The lack of formation of compound **57** from the oxidation of **55** was however surprising, as this compound was present throughout the initial incubation and the liberation of SDG **1** from **55** should have led to its formation. This lead to a re-analysis of the results from the AAPH **47**-mediated oxidation of SDG **1**. Compound **57** was seen to form rapidly (5 min) and then increase only gradually over the remainder of the 4 h incubation. This suggests several possibilities: **57** is an intermediate that forms other products or, **57** is an impurity present in the SDG **1** sample or forms from an impurity. Since none of the other major products found had undergone oxidation on both aromatic rings, it is unlikely that **57** leads to any of the major compounds, although it remains possible that unidentified minor or polymeric compounds were derived from **57**. While chromatographic techniques work the best for purifying lignans such as SDG **1**, it is extremely difficult to remove all impurities from SDG **1**. It is reasonable to speculate that compound **57** is an impurity or derived from an impurity in the SDG **1** sample. This is further supported by the observation that no other products were found in which oxidation had occurred on both aromatic rings.

5.12a*

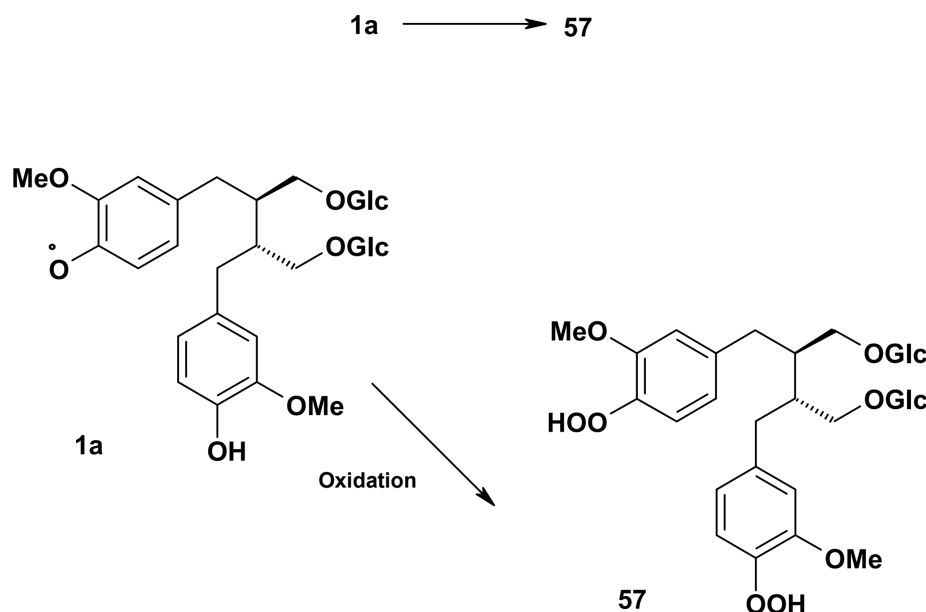


Figure 5.12. Proposed reaction scheme for the formation of compound **57**.
*For simplicity, Figure 5.12a shows the reactions by their product numbers.

A reaction scheme for AAPH-mediated SDG **1** oxidation which attempts to present an overall pathway to all of the oxidation products is shown in figure 5.13. An initial, resonance-stabilized phenoxyl radical would be formed and reactions at different positions on the aromatic ring would lead to the different products (Figure 5.13). **1a** leads to the formation of **56** as the result of trapping by AP. As indicated previously, it is however likely that **57** is not produced from AAPH **47**-mediated oxidation of SDG **1**, instead coming from a trace impurity in the SDG **1** sample. **1b** would lead to dimerization between C-5 positions of the aromatic rings to form the transient dimer **55**, and **1c** produces the BHT **17**-like dieneone addition product **58**.

Furthermore, the AAPH **47**-mediated SDG **1** oxidation produced a phenoxyl radical-derived product (**58**) that was not seen in the SECO **2** experiment. It is possible that formation of this product in the SECO **2** reaction was not observed due to the competing reactions at the primary alcohols (early forming products). If a longer incubation time were used, as was done by Eklund *et al*., analogous compounds to **58** may have been observed for SECO **2**. Finally, unlike the situation with SECO **2** oxidation, there were no early forming products derived from alkoxyl radical formation and no evidence for oxidation of any of the hydroxyl groups on glucose. This suggests that oxidation of the aliphatic hydroxyl groups of SECO **2** is unique.

Although Eklund *et al*., study did not include the AAPH **47**-mediated oxidation of SDG **1**, it did include oxidation of matairesinol.[152] Although the solubility properties would be different, neither compound has free primary alcohol groups on the lignan aliphatic chain, so they should undergo similar reactions. In Eklund *et al*., study matairesinol was found to only form products from oxidation of the phenol group (AP adduct, dimethyl furanone, C-5 dimer, quinone methide product, hydroxymatairesinol). While Eklund *et al*., results seem to agree with the results of this study, it remains inconclusive as SECO **2** also produced the same reaction products as matairesinol in Eklund *et al*., study.

5.13a*

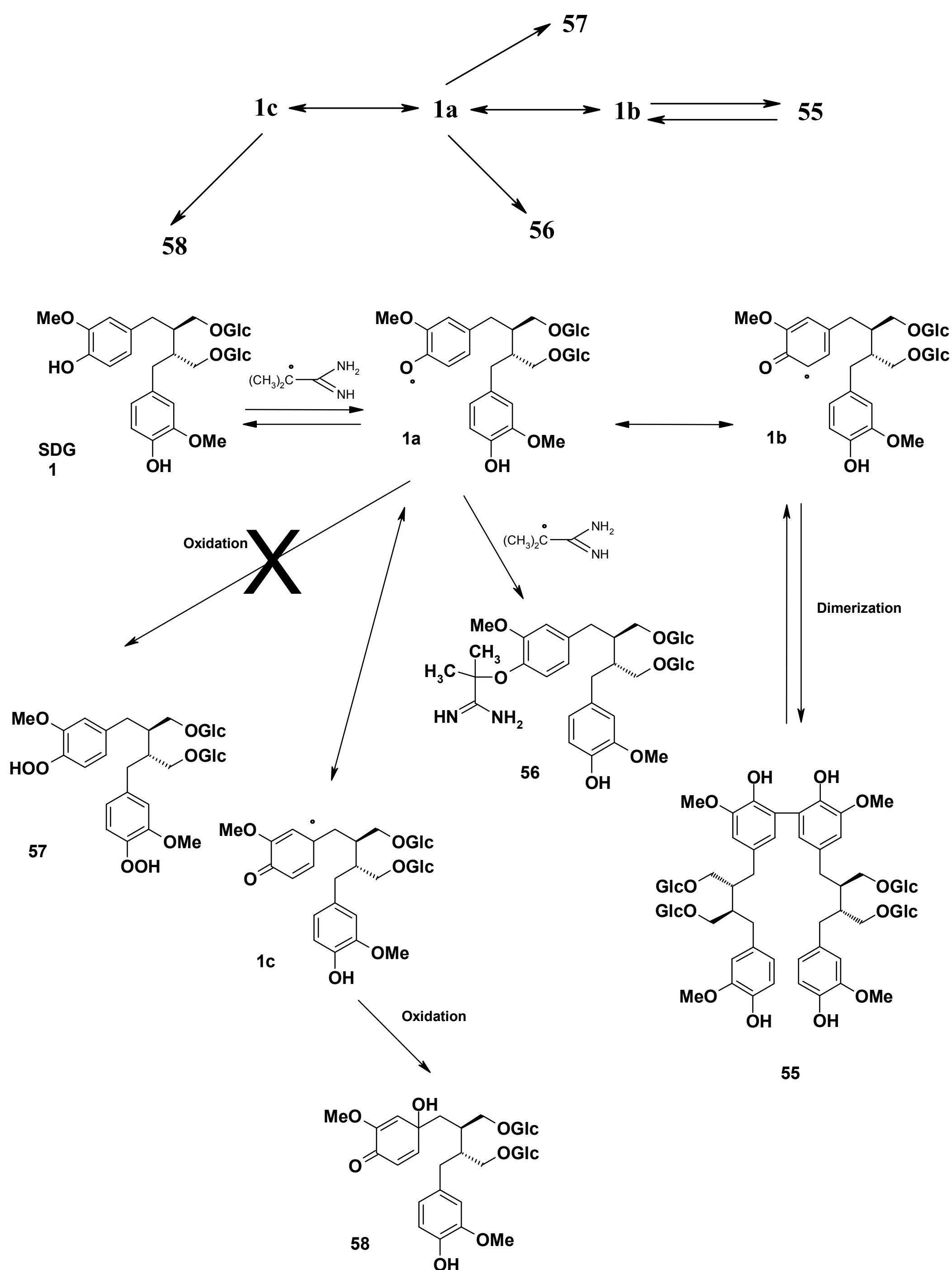


Figure 5.13. Proposed reaction scheme for SDG **1**:AAPH **47** oxidation
*For simplicity, Figure 5.13a shows the reactions by their product numbers.

Using BHT **17** as a model, the antioxidant stoichiometry of SDG **1** could be predicted to be as high as four. As was the case for SECO **2** the actual stoichiometric value (1.1-1.2) was much less. Part of this can be attributed to only one phenol ring reacting with AAPH **47**, as was also observed for SECO **2**, thus reducing the maximum stoichiometry to two. The antioxidant stoichometry of SDG **1** may be less than SECO **2** for several reasons: decreased lipid solubility and the lack of free primary alcohols on the lignan carbon chain to contribute to the antioxidant reactions. Consideration of the antioxidant contributions from the different products observed in this study would aid in rationalizing the stoichiometry observed. Compound **55** would be expected to scavenge two radicals per molecule and compound **56** would scavenge one radical per molecule. The stoichiometry for the other two products is less straightforward. Compound **58** is likely to have a stoichiometry of two as it is analogous to the reaction observed for BHT **17**. The major contribution to antioxidant stoichiometry would be from **56**. Contributions from **58** would not decrease the stoichiometry, whereas contributions from **55** would decrease the overall stoichiometry. The contribution from **57** cannot be determined due to the uncertainty associated with its origin.

Compound **56** was the major oxidation reaction product of SDG **1** with an ability to scavenge AP radicals during oxidation, thereby confirming SDG **1** as a chain-breaking antioxidant. The same observation was found for compound **50** in the SECO **2** oxidation study (section 4.3).

This study demonstrated that the flaxseed lignans SECO **2** and SDG **1** act as antioxidants in a homogeneous aqueous system. As well, this data clarifies the mechanism by which SECO **2** and SDG **1** scavenge AP radicals. In addition, this report shows that SECO **2** oxidation occurs on the aromatic (4-OH) and aliphatic (9-OH) hydroxyl protons. Conversely for SDG **1**, only compounds derived from the oxidation of aromatic hydroxyl protons were obtained because the 9-OH position is glucosylated. These results are important for better understanding about the chemistry behind flaxseed antioxidant activities. This study provided useful evidence that flaxseed lignans can be used as natural antioxidants, and may have roles in the prevention of oxidative stress and inhibitory activity against tumorigenesis similar to those found for flavonoids.[242,244,254]

5.5. The influence of the oxidation products on the antioxidant stoichiometry (*n*-value) of SDG 1 and SECO 2

Two possible antioxidant reaction pathways are shown based on the known reactions of BHT **17** (Figure 5.14-5.15). In the first step, SECO **2**/SDG **1** donates one hydrogen from a phenol hydroxyl group to a free radical to form the resonance stabilized phenoxy radical **2c**. In the second step, the intermediate **2c** scavenges a free radical to form non-radical compounds. In figure 5.14, this result in the radical adding to the phenol oxygen, in figure 5.15 the radical adds para to the phenol oxygen. The second aromatic hydroxyl group may undergo the same reactions to trap two additional free radicals.

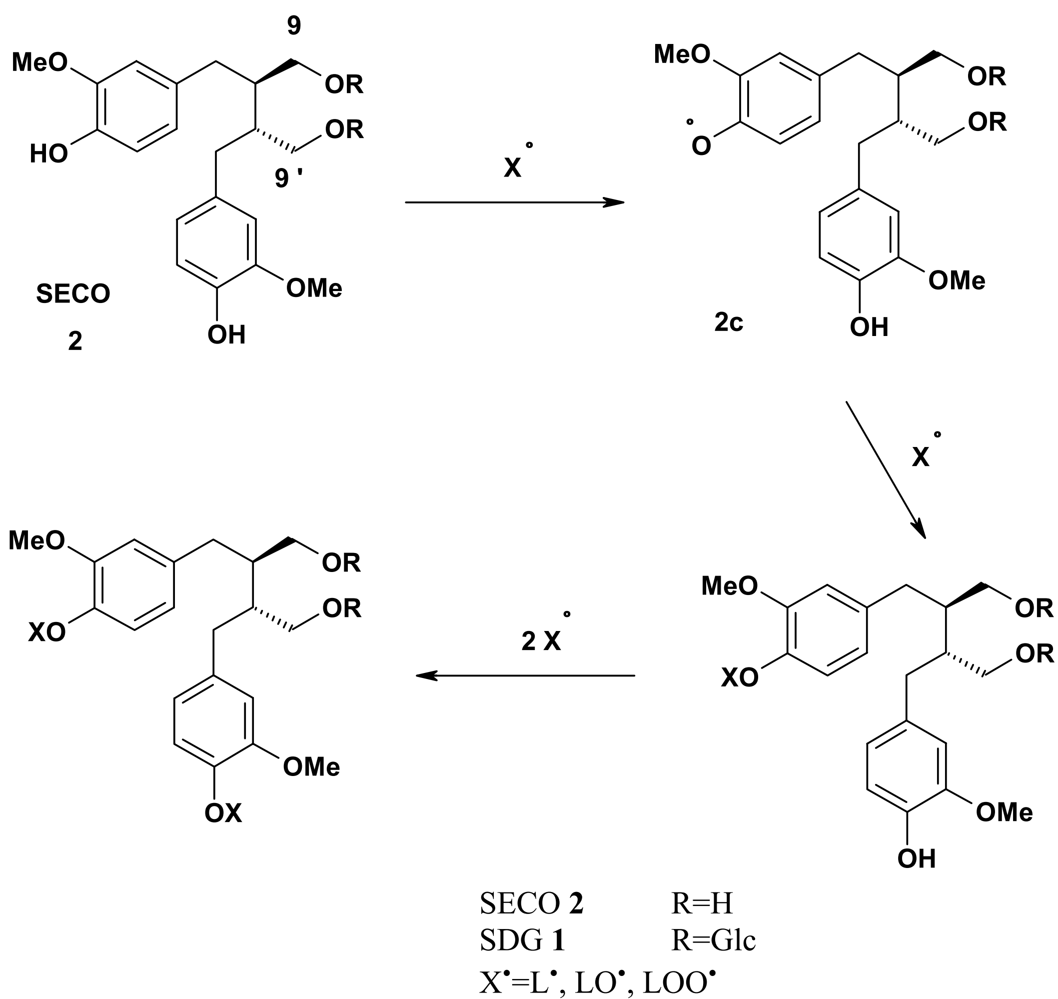
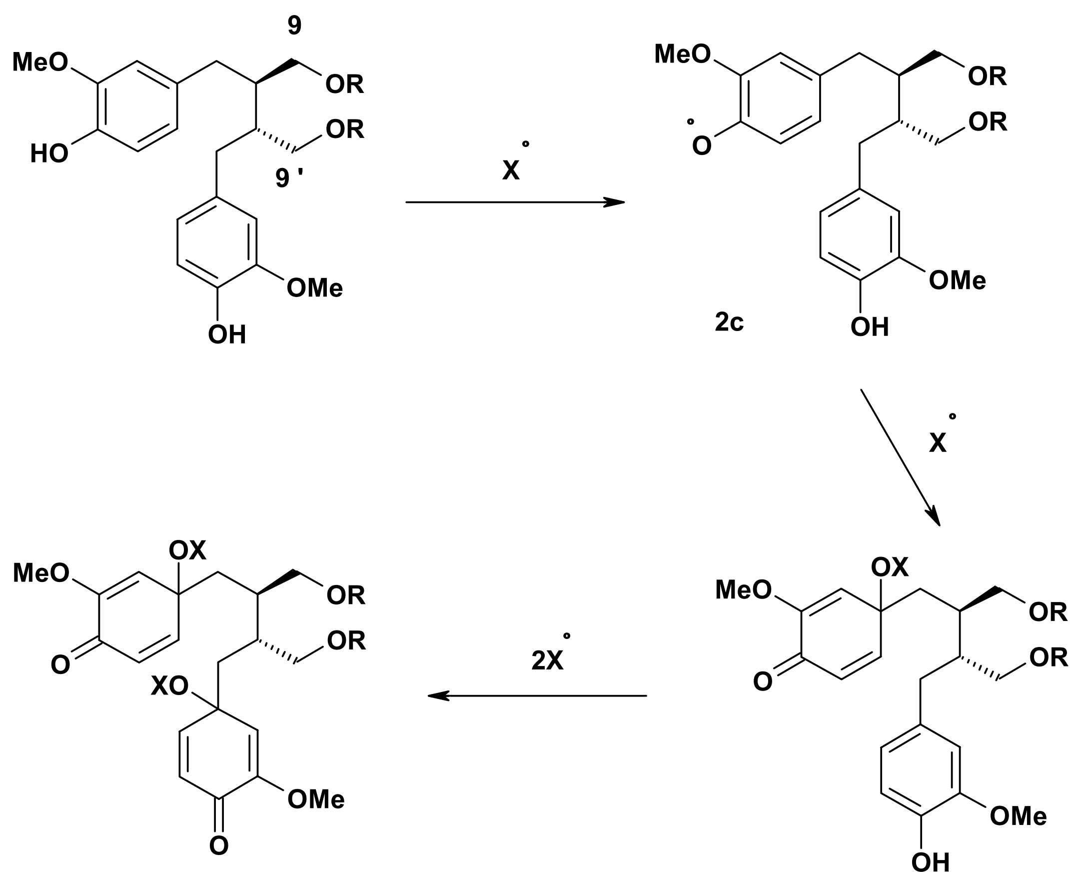


Figure 5.14. First predicted pathway for biological antioxidant activity of SECO **2** and SDG **1**.



SECO **2** R=H
SDG **1** R=Glc
X•=L•, LO•, LOO•

Figure 5.15. Second predicted pathway for biological antioxidant activity of SECO **2** and SDG **1**.

Compound **50** and compound **56** partially fit the predicted model about how SECO **2** and SDG **1** may react as antioxidants. This study demonstrated that the major oxidation products for both SECO **2** and SDG **1** have only one aromatic hydroxyl group involved in the oxidation. SDG **1** displays products that could be derived from both pathways, whereas SECO **2** only has products derived from the reactions shown in figure 5.14. Oxidative reactions in only one of the phenol rings

clearly decreases the potential stoichiometry for the molecule. It would seem that the oxidation products are resistant to further oxidation. This could be the result of an intramolecular interaction between the oxidized and reduced phenol rings which somehow prevents further oxidation, although this would require further investigation. Since no functional groups are introduced near the unreacted phenol, inhibition of phenol oxidation is not likely an electronic effect.

It is not clear why the antioxidant stoichiometry for SDG **1** and SECO **2** in this model system is far below the predicted ideal value. Both lignans tested seem to have a stoichiometric ratio of n=1 per two phenol OH groups. When DDPH scavenging was used as a measure of lignan stoichiometry, the stoichiometric factor for SECO **2** was 4.5 versus 1.9 for BHT **17**, which more closely agrees with the predicted stoichiometry for SECO **2**.[152] Reaction with the stable DPPH radical may be a better measure of the total number of easily reduced hydrogen atoms per molecule than the present method.

The results in this study suggest that the difference in antioxidant stoichiometry between SDG **1** and SECO **2** is not dependent on the difference in solubility but on the participation of the primary alcohol groups on SECO **2** in the antioxidant reactions. There are several lines of evidence to support this hypothesis. For example, from Eklund *et al*. study, the ratio of the stoichiometric results from DDPH scavenging for SECO **2** (4.5) as compared to matairesinol (2.9) is 1.55. [152] This is comparable to the ratio for the stoichiometric ratios found in this thesis for SECO **2** and SDG **1** (1.36-1.25). Matairesinol is more lipophilic than SECO **2**, but

does not have the two primary alcohol groups present. The product studies performed in this thesis indicate that reactions of the primary alcohol groups on SECO **2** lead to oxidation products which have stoichiometric ratios between two and one. Thus, a stoichiometric value of 0.3-0.4 could be assigned to the pair of primary alcohols in SECO **2**. Another study of antioxidant activity using the Ferric Reducing/ Antioxidant Power assay demonstrated that SECO **2** was better than matairesinol (1.2 vs 1.1) and END **12** was better than ENL **13** (0.2 vs 0.1).[306] In both cases primary alcohols were absent in the weaker antioxidant .

The only study which contradicts these results is one in which the inhibition of lipid peroxidation in a linoleic acid peroxidation assay by 10μM SDG **1**, END **12** and ENL **13** show % inhibition results of 31.3, 20.8 and 31.6%, respectively.[151] That is, the antioxidant activity is less for the compound with free primary alcohols (END). However, the % inhibition at 100μM SDG **1**, END **12** and ENL **13** was 58.7, 55.2 and 39%, respectively, with the most lipophilic compound (ENL), now showing the poorest antioxidant capacity and END **12** (free primary alcohols) being comparable to SDG **1**. This study also demonstrated that ENL **13** and END **12** were superior scavengers of hydroxyl radical compared to SDG **1**. The authors suggested that steric hindrance from the glucose groups on SDG **1** may interfere with radical scavenging by the phenol hydroxyls, a possibility that cannot be discounted as a contributing factor for the results found in this thesis.

It would be useful to determine how the aliphatic hydroxyl groups on SECO **2** contribute to antioxidant reactions by blocking the two hydroxyl groups in the

aromatic rings. Under acidic condition, SECO **2** converts to anhydro-SECO via the dehydration reaction and the formation of a furan ring.[32] Determination of the stoichiometric ratio of anhydro-SECO would indicate what role the aliphatic hydroxyl groups contribute to the antioxidant activity of SECO **2**. Alternatively, protection of the primary alcohols of SECO **2** with methoxy groups could also be used to probe the effect of the alcohol groups. Another analogue of SECO **2**, dihydroguaiaretic acid (CH_3 instead of CH_2OH) may also be an appropriate compound.

5.6. Estimation of antioxidant capacity (*n*-value) of SECO 2 oxidation products

The *n*-value of SECO **2** and SDG **1** oxidation products (as chain breaking antioxidants) can be estimated based on the ability to remove a free radical or to donate one hydrogen.[226] Based on this rule, compounds **50** and **51** would be predicted to have *n*=2 due to first, losing hydrogen (*n*=1) from SECO **2** molecule and second, trapping AP free radical (*n*=1). The two dimers **48** and **49** would have *n*=1 due to the lost of one hydrogen form SECO **2** molecule that undergoes dimerization. Compound **52** with peroxide in the structure would estimate to have *n*=0 because it does not lose any hydrogen or trap any AP free radicals.

5.7. Estimation of antioxidant capacity (*n*-value) of SDG 1 oxidation products

Compound **55** would be predicted to have n=1 due to the loss of one hydrogen from SDG **1** molecule that undergoes dimerization. Like compound **50** from SECO **2** oxidation, compounds **56** from SDG **1** oxidation would have n=2 due to losing one hydrogen and trapping an AP radical. Compound **58** would have n=2 due to first, losing hydrogen for the formation of a double bond (n=1) and, then, the addition of hydrogen (n=1) for the formation of a quinone molecule.

It would be useful to determine how aliphatic hydroxyl groups contribute to oxidation by blocking the two hydroxyl groups in the aromatic rings. Under acidic condition, SECO **2** converts to anhydro-SECO via the dehydration reaction and the formation of a furan ring.[32] Determination of the stoichiometric ratio of anhydro-SECO would indicate what role the aliphatic hydroxyl groups contribute to the antioxidant activity of SECO **2**. Anhydro-SECO would be expected to exhibit similar solubility in a liposomal system.

6.0. CONCLUSION

The major objectives of this study were to investigate the antioxidant properties and major oxidative compounds of SECO **2** and SDG **1**, using the AAPH **47** *in vitro* model of lipid peroxidation. AAPH **47** produces carbon-centred AP radicals upon thermal decomposition and provides a good model to study the antioxidant reactions of SECO **2** *in vitro*.

SECO **2** and SDG **1** were oxidized at 60°C by AAPH **47** for 5h and 4 h, respectively. The reaction mixtures were subjected to preparative and semi-preparative HPLC to yield five major oxidation products (**48**-**52**) for SECO **2** and three to four major oxidation products (**55-58**) for SDG **1**. The structure of these oxidation products have been identified using NMR and ES-MS.

This study demonstrated that the flaxseed lignans SECO **2** and SDG **1** act as chain breaking antioxidants in a homogeneous aqueous system. The antioxidant mechanisms of both SECO **2** and SDG **1** would be based on hydrogen donation ability of SECO **2** and SDG **1** to trap AP free radicals and consequently inhibiting radical chain propagation. SECO **2** oxidation occurs on the aromatic (4-OH) and aliphatic (9-OH) hydroxyl protons. Conversely for SDG **1**, only compounds derived from the oxidation of aromatic hydroxyl protons were obtained because the 9-OH position is glucosylated. After donating a hydrogen atom, SDG **1** forms resonance-stabilized phenoxyl radicals that form mainly stable non-radical compounds, which

do not easily participate in other radical reactions. The only exception is a dimer, which slowly decomposes during oxidation. SECO **2** also forms resonance-stabilized phenoxyl radicals that lead to stable, non-radical compounds. The stability of a SECO **2** dimer analogous in structure to the SDG **1** dimer could not be determined. SECO **2** however also forms alkoxyl radicals that appear to form mainly stable non-radical compounds. The one exception is a peroxyl linked dimer which decomposes during oxidation, although it appears to lead to the formation of the other stable, non-radical products.

Phenol oxidation products formed in both reactions were generally consistent with those observed in a recent report.[152] However, SECO **2** forms unique products that appear to be the result of alkoxyl radical formation, that were not observed by Eklund *et al*.

Two oxidation model systems were utilized to determine the potential for SECO **2**, SDG **1** and SDG polymer **3** to act as antioxidants in oil-based foods. A Rancimat was used for the measurement of inhibition of autoxidation mediated by flaxseed lignans in comparison with BHT **17**-mediated inhibition of autoxidation of canola oil. This study demonstrated that the lignans could produce a concentration-dependent inhibition of autoxidation, although none of the lignans was as effective as BHT **17**. In addition, SECO **2** was a superior antioxidant to SDG **1** and SDG polymer and SDG polymer was at least comparable to SDG **1**. SDG polymer may be a good candidate for a food antioxidant due to its lower cost. Second, a stoichiometric ratio measuring the ability of lignans to inhibit AAPH **47**-mediated

lipid peroxidation in a liposomal system was determined.[227] The stoichiometric ratio for SECO **2** and SDG **1** were 1.5 and 1.1-1.2, respectively compared to BHT **17** (2.0), signifying that both SECO **2** and SDG **1** delayed AAPH **47**-induced lipid autoxidation. As well, SECO **2** and SDG **1** from flaxseed lignans are chain-breaking antioxidants.

Both model systems showed that as an antioxidant, SECO **2** provides better protection than SDG **1**. The observed induction times were consistent with the calculated stoichiometric ratio, and illustrative of two appropriate oxidation models. The greater antioxidant activity of SECO **2** vs SDG **1** is proposed to be the result of the additional contributions to radical scavenging by the alkoxyl radical-derived products found for SECO **2**. Most other examples of lignan antioxidant capacity in the literature appear to agree with this hypothesis. In addition, the antioxidant stoichiometry is approximately 50% that predicted from the number of phenol OH groups per molecule, suggesting that calculating relative antioxidant potential in this manner is not appropriate for the lignans.

Although SECO **2** and SDG **1** are less potent antioxidants compared to BHT **17**, they have potential advantages over BHT **17** in that they are natural antioxidants that have potential health benefits associated with their consumption.[129,153,277] These results provide better understanding of the chemistry behind flaxseed antioxidant activities and suggest that flaxseed lignans may be used as natural antioxidants.

7.0. FUTURE RESEARCH

Future biological studies require confirmation of SECO **2** as a chain breaking antioxidant in prevention of lipid oxidation, protein oxidation and DNA oxidation associated with oxidative stess. Other *in vitro* studies with animal and human hepatic microsomal preparations could use these compounds to investigate their antioxidant actions. Tissue culture of animal and human cancer cells could be a useful way to examine whether these compounds prevent growth of cancer cells.

Further investigation on the differing antioxidant activities between SECO **2** and SDG **1** (*in vivo* study) would be enlightening because most of the glycosides would be converted to aglycones by intestinal microorganisms.[286] In addition, it may be beneficial to treat the flax products in a way that would convert SDG **1** to SECO **2** in order to increase its antioxidant capacity.

This study demonstrated that SECO **2** and SDG **1** act as antioxidants in homogeneous solution. The mechanism by which SECO **2** and SDG **1** scavenges AP radicals have been clarified. However, the details of the behaviour and action of SECO **2** and SDG **1** in the membranes remain to be established. Further areas of potential study include:

1. Determine how the primary aliphatic hydroxyl groups of SECO **2** contribute to its antioxidant effect. This could be accomplished by studying structural analogues in which the alcohols have been removed.

2. Determine whether the mechanism of the inhibition of oxidation by SECO **2** and SDG **1** in cell membranes is the same as those in homogeneous solutions. Microsomal lipid peroxidation is one possible method that could be used to assess this. The stable dimers may be useful as potential markers of lignan antioxidant activity in these sysytems.

3. Phospholipids spontaneously form bilayers called liposomes when dispersed in water, and these can serve as good models for real biological membranes. It remains to be determined whether the kinetics and mechanisms for the oxidation of lipids in liposomal membranes are the same as those of phospholipids and fatty acids in organic solution.

4. Further toxicological and safety profiles need to be determined in studies of chronic exposure.

5. Determine whether SECO **2** and SDG **1** can act as preventive antioxidants.

6. Determine whether SECO **2** and SDG **1** exhibit a synergistic effect *in vitro*.

7. Determing the ability of SECO **2** and SDG **1** to act as antioxidants in different emulsion food systems (oil in water, water in oil).

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