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Isolation, Synthesis, and Metabolism of Polyphenols: Stilbenoids, Gallotannins and Ellagitannins

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ISOLATION, SYNTHESIS, AND METABOLISM OF POLYPHENOLS:

STILBENOIDS, GALLOTANNINS AND ELLAGITANNINS

BY

DANIEL B. NIESEN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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OF

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2016

ABSTRACT

The roles of dietary polyphenols, such as resveratrol found in red wine and grape seed extract, have become an increasingly more popular research topic. Dietary polyphenols have even achieved media success for their potential benefits on human health and disease. The success is frequently attributed to the compounds' antioxidant and reactive oxygen species (ROS) scavenging abilities. Not only are polyphenols highlighted as the beneficial health constituents of herbal remedies, but there are also two plant-polyphenol drugs approved by the United States Food and Drug Administration, Crofelemer (a proanthocyanidin oligomer from *Croton lechleri*) and Veregen (catechins from *Camellia sinensis*). Due to the demand for novel polyphenolic lead compounds, three major classes were chosen to be explored as part of this dissertation: stilbenes, gallotannins, and ellagitannins. The investigation of stilbenes includes an invited literature update on novel natural stilbenes, which have been isolated and identified since 2009. The review concludes by discussing the biological activities and potential health benefits of this polyphenol sub-class. Additionally, two new monomeric stilbenes, namely, vulpinoideols A and B, along with ten known compounds were isolated from a fox sedge (*Carex vulpinoidea*) acetone seed extract. All of the isolates were evaluated for their tyrosinase enzyme inhibitory activity, along with the well-known competitive inhibitor, arbutin. A synthetic route for naturally isolated gallotannins, namely, maplexins F and J, previously isolated from red maple (*Acer rubrum*) tree bark, was developed. Additionally, the ligand-enzyme interaction between the gallotannins and their target enzyme, α -glucosidase, was evaluated using

biophysical tools including isothermal titration calorimetry (ITC), fluorescence, and circular dichroism (CD) spectroscopic methods. Finally, a synthetic route was also developed for the ellagitannin gut-microbial metabolites, urolithins (dibenzopyranone derivatives), along with analytical UFLC-MS/MS methods to study the cellular and tissue uptake of the urolithins, as well as other dietary polyphenolic metabolites of interest.

ACKNOWLEDGMENTS

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Additionally, I would like to thank my girlfriend and continued support-system, Nicole Campano, and our newest four-legged addition, Lincoln. As this chapter comes to a close, I cannot wait to see what adventure is next. I would like to thank my lab members and colleagues, especially Dr. Alvin C. Bach II, Dr. Hang Ma, and Craig Hessler, three friendships that I know will continue into the future. Without the love and support of Nicole, my parents, my advisor, and my friends, this work could not have been possible. It means so much to have all of you close by.

PREFACE

This dissertation has been written in manuscript style format.

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STATEMENT OF THE PROBLEMS

Natural products continue to provide an inspiration for new drug targets and synthetic scaffolds. Between 1940 and 2010, a total of 175 small-molecule, anti-cancer therapeutics have been approved by the Food and Drug Administration. Of those 175 approved therapies, 49% were either discovered or derived directly from a natural product [1]. Even with natural products providing such a large influence towards the generation of new pharmaceuticals, *there is still a growing demand for the discovery of new lead compounds*. This demand puts continuous pressure on finding novel compounds, and sources, for drug candidates.

The polyphenols of interest are secondary metabolites, and are not directly responsible for the growth and development of the producing organism. As a result, these compounds are frequently isolated in low yields from their natural sources. Obtaining the natural biomass can be economically and scientifically impractical (e.g. deep ocean bacteria or the bark of a tree). *Therefore, to investigate a natural product isolate as a potential drug candidate, a synthetic route must often be developed*.

Over the past decade, the fate of dietary polyphenols upon ingestion has continued to attract increased research attention. Several sub-classes of polyphenols are poorly bioavailable in their intact forms, but are extensively metabolized by gut-microflora. This results in the hydrolysis of sugar substituents, alkylation of vulnerable oxygen and nitrogen functional groups, and a series of oxidation-reduction reactions. The final metabolite of dietary polyphenols often has a completely different chemical structure

and set of properties from what was originally consumed. As these metabolized polyphenols are, in fact, the biologically available metabolic endproducts, *it is imperative to develop analytical methods for evaluating the potential impact of these compounds.*

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CHAPTER 1

STILBENES

Manuscript 1 appears as published:

Invited Review: Beyond Resveratrol: A Review of Natural Stilbenoids Identified from 2009-2013. Niesen, Daniel B.; Hessler, Craig; Seeram, Navindra P. (2013) *Journal of Berry Research*. 4, 181-196.....8

Manuscript 2 appears as published:

Phenolic Constituents of *Carex vulpinoidea* Seeds and their Tyrosinase Inhibitory Activities. Niesen, Daniel B.; Ma, Hang; Yuan, Tao; Bach, Alvin C. II; Henry, Geneive E.; Seeram, Navindra P. (2015). *Natural Product Communications*. 10, 491-493.....38

ABSTRACT

Natural stilbenes have become an increasingly popular research topic of many natural product groups throughout the world, due to their elaborate structures and dynamic biological activities. Stilbenes are phenolic compounds, and are an exciting class that have been found in 33 plant families. Even though their carbon monomeric form is a simple 1,2-diphenylethylene unit, stilbenes display a wide variety of polymerization and oligomer construction [1]. They most commonly refer to the compounds that are hydroxylated derivatives of the stilbene carbon backbone. Stilbenes have been isolated in monomeric forms, like resveratrol, oligomer forms, such as α -viniferin, and glycosides like astringin [1].

Stilbenes are produced as a woody metabolite, and may serve as constitutive and inductive defense agents. The antimicrobial activity of plant stilbenes, and the fact that they are both constitutive and inducible, strongly suggests that their concentrations are a good indication of their role in disease resistance [1]. Because of this, stilbenes have been the focus of a variety of research topics for their potential benefits.

Resveratrol has been extensively studied due to its wide range of biological activities and occurrence in plant foods, including grape and some berries. Apart from the intact resveratrol molecule and closely related analogs, this compound can be regarded as a monomer which occurs as a primary building block for subsequent polymerization, which leads to the extensive structural diversity within the chemical classification.

Stilbenoids exhibit a vast array of polymerization and oligomeric construction, with over 60 such naturally occurring stilbenes being isolated and identified in the last five years alone, adding to the hundreds which are already known to date (Manuscript 1).

The *Carex* genus (family, Cyperaceae) contains over 2000 species [2], but the majority of these remain largely uninvestigated for their phytochemical constituents. This is unfortunate, considering that the Cyperaceae family is one of the few plant families known to produce stilbenoid derivatives [3, 4]. Previously, our group has studied *Carex* species found in the northern regions of the United States, which has yielded several bioactive phenolic constituents, including resveratrol oligomers and other stilbenoids [5, 6].

Hyperpigmentary disorders, such as melasma and freckles, are due to the abnormal accumulation of melanin. The tyrosinase enzyme has been implicated as a key enzyme in melanogenesis, as it initiates an enzymatic cascade through the conversion of tyrosine and 3, 4-dihydroxyphenylalanine (L-DOPA) into DOPA-quinone [7, 8]. Notably, plant natural products, in particular, phenolic compounds, have shown great promise as tyrosinase inhibitors, a primary example being the phenolic glycoside, arbutin, which is widely used for commercial cosmetic applications [9, 10]. Moreover, several phenolic sub-classes, including flavonoids, chalcones, and stilbenoids, are known to be tyrosinase inhibitors [11-13].

In the pursuit to of novel stilbenoids, the seeds of the previously uninvestigated *Carex vulpinoidea* Michx were investigated for their phytochemical composition. The isolates were also evaluated for their anti-tyrosinase activities. The approach involved isolation and structural elucidation of phytochemicals by using various chromatographic separation techniques and analytical approaches including NMR (nuclear magnetic resonance) spectroscopy and MS (mass spectrometry).

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MANUSCRIPT 1

Manuscripts 1 appears as published in the Journal of Berry Research.

Invited Review:

Beyond Resveratrol: A Review of Natural Stilbenoids Identified from 2009-2013.

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Abstract

Polyphenols constitute a large chemical class of phytochemicals among which the stilbenoid sub-class has attracted significant attention due to their elaborate structural diversity and biological activities. Resveratrol, a well-known stilbene, has been extensively studied due to its wide range of biological activities and occurrence in plant foods, including grape and some berries. Apart from the intact resveratrol molecule and closely related analogs, this compound can be regarded as a monomer which occurs as a primary building block for subsequent polymerization which leads to extensive structural diversity. Consequently, stilbenoids exhibit a vast array of polymerization and oligomeric construction, with over 60 such naturally occurring stilbenes being isolated and identified in the last five years alone, adding to the hundreds which are already known to date. This review updates the literature on natural stilbenoids which have been isolated and identified since 2009 and discusses the biological activities of this sub-class of bioactive polyphenols as a whole.

1.0 Introduction

1.1. Stilbenes

Over the past few decades, significant research attention has been directed towards the investigation of polyphenols, a large class of secondary metabolites which are abundant in plants and plant-derived foods including grapes, and some berries and nuts. Within the large chemical class of (poly)phenolic compounds, the stilbenoids, which occur in 33 plant families, have been extensively studied both as pure compounds and enriched plant derived extracts [1, 2]. Consequently, their potential applications either as botanical supplements or as active constituents in medicinal and cosmetic preparations have been evaluated [1]. Notably, stilbenoids have been isolated and studied as monomers and oligomers, as well as glycosylated derivatives with the best known example being resveratrol as well as others including α -viniferin, and astringin (see Fig. 1) [2]. The carbon skeleton of stilbenes occurs as a C6–C2–C6 unit, namely, a 1, 2-diphenylethylene moiety, however, the commonly hydroxylated derivatives provide the class with a wide variety of polymerization and oligomeric construction. Stilbenes are produced by plants as a woody metabolite, as well as constitutive and inductive defense agents. The antimicrobial activity of plant stilbenes, and the nature of these compounds as being both constitutive and inducible secondary metabolites, suggests that their *in situ* concentrations are a good indication of disease resistance [2]. Historically, significant research has focused on the role of stilbenes and their activity as anti-bacterial agents, antioxidants, anti-inflammatory agents, anticancer and cancer chemopreventive agents, and more recently their role in the regulation of several human degenerative diseases [3-9].

1.2. Occurrence of resveratrol in berries

The occurrence of stilbenes in berries has largely been reported for the so called ‘model stilbenoid’ compound, namely, resveratrol (Fig. 1) which was originally isolated from the roots of *Veratrum grandiflorum* in 1940 [10]. Notably, as mentioned previously, in nearly every case highlighted in the current review, resveratrol has been used as the primary building block during the polymerization into larger stilbenes.

Figure 1. Stilbene derivatives highlighting the common monomer, resveratrol (left), a trimer α -viniferin (center), and a glycosylated derivative, astringin (right).

In many grape varieties, *trans*-resveratrol is a phytoalexin produced to combat the growth of fungal pathogens such as *Botrytis cinerea*, a necrotic fungus whose most notable host is wine grapes [11]. Resveratrol’s presence in *Vitis vinifera* grapes is also constitutive, with a natural accumulation in the skin of ripe berries. In muscadine grapes (*Vitis rotundifolia*), a species native to the southeastern United States, resveratrol has been isolated from the seeds as well as the skin [12]. The concentration of resveratrol present in grape skins varies with the grape cultivar, its geographic origin,

and exposure to infections. As red wine is fermented with the skins, it contains a higher concentration of resveratrol as compared to white wines. Depending on the grape variety, the concentration of resveratrol in red wines ranges between 0.2–5.8mg/L, with a direct correlation in the amount of fermentation time a wine spends in contact with the grape skins [13].

Resveratrol has also been detected in several other berry varieties. It has been identified in blueberry, bilberry, cowberry, red currant, cranberry and strawberries; however these berries contained less than 10% of that present in grapes [14, 15]. The content of trans-resveratrol in the fresh weight of the above fruit ranges from 3–30µg/g [15]. Additionally, it has been reported that heating and cooking the berries will contribute to the degradation of resveratrol [14].

1.3. Occurrence of resveratrol and other stilbenes in non-berry food sources

Resveratrol and polymeric stilbenes have been found in other food sources apart from the various berry varieties. Interestingly, a source of resveratrol derivatives is peanuts (*Arachis hypogea*), in particular, sprouted peanuts, where the resveratrol content rivals that of grape skins. Depending upon peanut cultivar, the resveratrol content ranges from 2.3–4.5µg/g before sprouting and 11.7–25.7µg/g after sprouting [16]. Two new resveratrol dimers were recently isolated from peanut seeds and are discussed later in this review [17]. Cocoa powder, baking chocolate, and dark chocolate have also been reported to contain small concentrations of resveratrol in normal consumed quantities i.e. 0.35–1.85mg/kg [18].

1.4. Previous review articles on stilbenes

To date, there are several reviews available on the pharmacological benefits of resveratrol and its analogs [19–21]. Most recently, in 2009, Shen and co-workers have published a comprehensive examination of the novel chemistry related to stilbenes discovered over the time period from 1995 to late 2008 [2]. Therefore, in this review, we examine new stilbene chemistry that has been reported from 2009 to late 2013, bridging the gap in period previously reported by Shen and co-workers [2]. During the current period covered herein, over 60 naturally occurring stilbenes have been isolated and identified with structures ranging from glycosylated monomers to a hexamer. Current scientific interest in determining the biological activity of this class of compounds will also be highlighted.

2. Stilbenes isolated and identified since 2009

2.1. Compound classification

While more complex classification systems for stilbenes have been proposed by Sootheeswaran and Pasupathy [22], for the purpose of this review, the novel chemistry isolated has been grouped into: monomers, dimers, trimers, tetramers, and hexamer units. Additionally there was one norstilbene isolated, as well as stilbenes containing glycoside moieties, which we have differentiated based on their carbon-carbon or carbon-oxygen connectivity. These compounds are discussed below.

2.2. Stilbene monomers

Only two stilbene monomer derivatives were isolated during this time period. While the stilbene structure provides a large variety in the ways of polymerization, the diphenylethylene unit does not have many locations for potential modifications into novel monomers. Although the discovery of new monomers is less common, two prenylated derivatives were successfully isolated and identified. From black skin peanut seeds challenged with the fungal strain *Rhizopus oligoporus*, Liu et al. [23] isolated the methoxy-prenylated derivative shown in Fig. 2 (compound shown on left). Shan and co-workers [24] successfully isolated the additional monomer, cudrastilbene, from the roots of *Cudrania tricuspidata*, an ethnobotanical plant used commonly in China, Korea, and Japan for medicinal purposes. The two new compounds can be found in figure 2.

Figure 2. 3,5,3'-trihydroxy-4'-methoxy-5'-isopentenylstilbene (left) and cudrastilbene (right) isolated from fungal challenged black skin peanut seeds and *Cudrania tricuspidata* respectively.

2.3. Stilbene dimers

During the time period of this review, a total of 17 dimeric stilbenes were reported. Among the oligomeric stilbenes, the resveratrol monomer is most commonly used for construction. There are many combinations of C–C and C–O bonding patterns that can arise during the polymerization of the resveratrol units. However, the most common is

the formation of the benzofuran moiety. This is apparent, as it is seen in almost all of the newly isolated stilbene dimers.

Arguably, most structurally unique of the dimers are the two phytoalexins isolated by Sobolev et al. [17]. Interestingly, the production of these two compounds was induced by subjecting peanut seeds (*Arachis hypogaea*) to fungal (*Aspergillus caelatus*) infection. Isolation and purification of the fungal challenged seed's chemical constituents resulted in the discovery of two new prenylated dimers of resveratrol, arahypin 6 and 7 (Fig. 3).

Figure 3. Arahypin 6 (left) and arahypin 7 (right) isolated from peanuts (*Arachis hypogaea*).

Plants of the genus *Gnetum* have previously been reported to be rich sources of oligomeric stilbene derivatives [25]. Similarly, in the current time period of 2009–2013, for this review, this was also true with the isolation and characterization of macrostachyols C and D from the roots of *Gnetum macrostachyum* (Fig. 4) [25]. Interestingly, macrostachyol C does not contain a benzofuran moiety as observed in the majority of the stilbene dimers.

The genus *Shorea* had previously been reported to contain stilbenoids, although the work done by Patcharamun and co-workers in 2011 was the first phytochemical investigation conducted on *Shorea roxburghii* [26], a medicinal plant used in India. This work resulted in the isolation of roxburghiol A (Fig. 5).

Figure 4. Macrostachyols C (left) and macrostachyol D (right) isolated from *Gnetum macrostachyum*.

Figure 5. Roxburghiol A isolated from *Shorea roxburghii*.

Vaterioside A (Fig. 6), was isolated from the leaves of *Vateria indica* by Ito et al. [27]. Previously, studies have shown that this carbon skeleton can be produced by exposing ϵ -viniferin to photo-oxidative conditions [28]. However, this is the first chemical isolation from natural sources. A glycosylated form of vaterioside A was additionally isolated during this phytochemical investigation (compound is shown in Fig. 22).

Four new dimers were reported from the stem wood of *Hopea hainanensis* by Ge et al. [29]. This group has previously isolated, and characterized, several other stilbene oligomers from the *Hopea* genus. This recent work resulted in the isolation and characterization of four new structures, each of which has notable differences (Fig.7). Hopeahainol C is more unsaturated when compared to the other dimers of this review, lacking a single sp^3 hybridized carbon. The relative stereochemistry of these compounds were unambiguously determined using NOESY experiments [29].

Figure 6. Aglycone of vaterioside A isolated from *Vateria indica*.

Figure 7. Hopeahainols C-F (left to right, top to bottom, respectively) isolated from *Hopea hainanensis*.

Examination of the branches and twigs of the tropical plant *Vatica mangachapoiby* Qin et al. [30] afforded the three new dimers shown in Fig. 8. The compounds were isolated in a bioassay-guided fractionation targeting compounds with xanthine oxidase (XO) and acetylcholinesterase (AChE) inhibitory effects. Interestingly, vaticahainol A shows rearrangement from the original resveratrol unit. The structure contains a lactone moiety, which is not seen in the other dimers discussed in this review. Vaticahainol B contains a quinone ring, which also has not been seen in many other isolated stilbenes. The quinone moiety is recognized by four quaternary sp^2 C-atoms and two protonated sp^2 C-atoms, which suggests the presence of a cyclohexa-2,5-dienone system. The final structure, vaticahainol C, contains a distinctive phenanthrene moiety [30].

Figure 8. Vaticahainols A-C (left to right, respectively) isolated from *Vatica mangachapoiby*.

While studying the stems of *Vatica albiramis*, Abe et al. [31] isolated several new stilbenes oligomers [31]. These include the dimer, albiraminol B, which is nearly identical to a previously known compound, malibatol A (Fig. 9). The only difference between these two compounds is stereochemistry in the connectivity between the A1 and A2 rings. Notably, malibatol A was also isolated by this group from the same fraction.

Figure 9. Albiraminol B (left) and malibatol A (right) isolated from *Vatica albiramis*.

Working with the methanol extract of the whole plant *Cyperus longus*, which is used traditionally in Egyptian medicine as a tonic and a diuretic, Morikawa et al. [32], using bioassay guided fractionation, were able to identify three stilbenes (Fig. 10) [32]. These include longusol A and B which contain similar connectivity using the common benzofuran ring to connect the two resveratrol monomers. The carbon skeleton presented by these two structures has been previously reported as opposing stereoisomers, as well as re-isolated by this group during the investigation of *C. longus*. Longusol C contains a 1,4-dioxane moiety to connect the resveratrol units, however its stereoisomer was also previously reported. The stereoisomer was again re-isolated by the group.

While working with fungal challenged black skin peanut seeds Liu et al. [23] isolated two new prenylated stilbene dimers, arahypin-11 and arahypin-12, along with the monomer previously mentioned in Fig. 2. Their isolation of prenylated stilbenes from fungal challenged peanut seeds follows previous publications, most notably the previously mentioned Sobolev et al. [17]. Arahypin-11 and -12 can be found in Fig. 11.

Figure 10. Longusol A-C (left to right, respectively) isolated from *Cyperus longus*.

Figure 11. Arahypin-11 and -12 isolated from fungal challenged black skin peanuts.

The last dimer isolated contains an aryl coupling between two benzofuran stilbene monomers. The roots and stems of *Vitis amurensis* have been used ethnobotanically as pain relievers, and in this investigation yielded the isolation of the novel dimer [33]. The compound can be found in Fig. 12.

Figure 12. Dimer isolated from the roots and stems of *V. amurensis*. The compound contains an aryl carbon-carbon linkage of two benzofuran monomers.

2.4. Stilbene trimers

Of the compounds isolated during this time period, only 4 were stilbene trimers. Three of these four compounds were isolated from the plant *Paeonia suffruticosa* by He et al. [34], two of which are a pair of stereoisomers (Fig.13) [34]. The third compound, isolated from *P. suffruticosa* is *cis*-gnetin H. The three compounds contain the same carbon skeleton and the resveratrol monomer units are connected by benzofuran rings. The absolute stereochemistry of the three trimers was determined using NOESY NMR and circular dichroism [34].

Figure 13. *cis/trans*-suffruticosol D and *cis*-gnetin H (left to right, respectively) isolated from *Paeonia suffruticosa*.

As previously mentioned, plants of the genus *Gnetum* are rich sources of oligomeric stilbene derivatives [25]. Along with the isolation of the two dimers, macrostachyol C and D, Sri-in et al. isolated a novel trimer from *Gnetum macrostachyum*, named macrostachyol B (Fig. 14) [25]. The structure contains an interesting carbon bridge creating the bicyclic internal ring system.

Figure 14. Macrostachyol B isolated from *Gnetum macrostachyum*.

2.5. Stilbene tetramers

Similarly to the stilbene dimers, the majority of the stilbene tetramers contain a benzofuran moiety. This is due to the fact that the tetramers are primarily ‘dimers of

dimers'. As shown in the current review, as well as the most recent other review reported by Shen et al. [2], the diversity of stilbene dimers is quite extensive. Due to the number of active functional groups that allow for the ease as well as variety in polymerization, once a dimer is formed, there are several positions whereby these newly formed dimers can polymerize to form tetramers of resveratrol. This ease in polymerization is responsible for the increasing diversity of resveratrol dimers and tetramers.

Two new tetramers were isolated from *Upuna borneensis*, conducted by Ito et al. [35]. The group has previously reported a structural variety of resveratrol oligomers from *U. borneensis*, while in this report they were investigating the acetone extract of the plant's stems. Their work afforded the two new tetramers, upunaphenols O and P (Fig. 15) [35]. Upunaphenol O consists of the resveratrol dimers ampelopsin A and *cis*- ϵ -viniferin. Additionally both of the dimer-subunits were isolated by the group, whom had hypothesized that they are indeed the building blocks of the tetramer [35]. Upunaphenol P contains a similar dimer unit to upunaphenol O, however the determination of whether it is derived from ampelopsin A or B is inconclusive. Additionally, it contains a unique C–C bridge between the two dimer pieces.

Figure 15. Upunaphenol O (left) and Upunaphenol P (right) isolated from *Upuna borneensis*

Along with the isolation of the two dimers, macrostachyol C and D, and the trimer macrostachyol B, this group also isolated a novel tetramer from *Gnetum macrostachyum*, macrostachyol A (Fig.16) [25]. Macrostachyol A differs from other common tetramers, as based on the bonding pattern it does not appear to be a dimer of a dimer. Instead, the bonding suggests that macrostachyol A is derived from latifolol, a resveratrol trimer, combined with yet another resveratrol unit through oxidative coupling [25]. It is noteworthy to add that this group also isolated latifolol, which adds credibility of their oxidative coupling hypothesis.

Figure 16. Macrostachyol A isolated from *Gnetum macrostachyum*.

The next two resveratrol tetramers return to the commonality of a dimer of dimers and include cajyphenol A and B which were isolated from *Cayratia japonica* by Bao et al. [36] (Fig. 17). These two tetramers contains the same carbon skeleton, however differ in their attachment of the two dimer sub-pieces. Additionally the southern portion of each the two molecules contains opposing relative stereochemistry.

Figure 17. Cajyphenol A (left) and cajyphenol B (right) isolated from *Cayratia japonica*.

The final resveratrol tetramer isolated during the time of this review, is also the only tetramer that displays a carbon-symmetric structure (Fig. 18). Vateriaphenol F was isolated from the leaves of *Vateria indica* by Ito et al. [27]. The dimer of dimers is constructed from two ϵ -viniferin pieces. In this example, the use of the furan moiety has been used to polymerize each step of the oligomer from the resveratrol monomers.

Figure 18. Vatriaphenol F isolated from *Vateria indica*.

2.6. Stilbene hexamer

There was only one hexamer isolated during the time period covered by this review, and it is only the fifth instance of a resveratrol hexamer being isolated from a natural source (Fig. 19). This hexamer was isolated from the acetone extract of *Vatica albiramis* stems by Abe et al. [31]. The structure consists of a tetramer, vacticanol A, and a dimer unit. Additionally it is important to note the 1,2-aryl shift which occurred in resveratrol F has rarely been seen in isolated oligomers [31].

Figure 19. Albiraminol A isolated from *Vatica albiramis*.

2.8. O-glycosylated stilbenes

The previous stilbenes reported in this review have not been glycosylated. During the period in which this review covers, there were 13 O-glycosylated structures reported of which, four were monomers, six dimers, and three tetramers. Like many other classes of compounds, the most common sugar moiety added is glucose and in the case of this review, all of the sugar moieties added through O-glycosylation were glucose.

Upon fractionation of the aerial portions of the Mongolian medicinal plant, *Scorzonera radiata*, Wang et al. [37] isolated four new glycosylated monomers (Fig.21) [37]. These monomers differ from the typical resveratrol monomer with the addition of the acetate unit at C1, as well the substitution pattern in ring B. Additionally, several of these monomers have methyl modifications at various locations generating methoxyl functional groups. The group also isolated a dimer of Fig. 21's compound 1, which was connected through a C–C bond and the 5' carbon (Fig. 21) [37].

Figure 21. Four glycosylated monomers and a dimer isolated from *Scorzonera radiata*.

Two new glycosylated compounds were isolated in the leaves of *Vateria indica* by Ito et al. [27]. One of the structures, a dimer, is previously mentioned in this review as having an aglycone that had previously not been isolated in nature (Fig. 5). The group also isolated a tetramer with an aglycone, consistent with that of hopeaphenol, a dimer of two ampelopsin units (Fig. 22) [27].

Figure 22. Vateriaoside B isolated from *Vateria indica*.

Two publications from the same group, Abe et al. [31, 38], resulted in the final six O-glycosylated stilbenes isolated in the time frame of this review [31, 38]. All six were isolated from the stems of *Vatica albiramis* and named vatalbinosides A-F (Fig. 23). The group isolated 4 dimers and 2 tetramers. While these glycosylated versions are newly isolated compounds, each of the compounds respective aglycones are previously described skeletons.

Figure 23. Vatalbinsides A-F isolated from *Vatica albiramis*.

2.9. C-glycosylated stilbenes

While O-glycosylated structures are much more common, occasionally C-glycosylated structures are also reported among stilbenoids. Abe et al. [39] isolated four new C-glycosylated structures from *Hopea parviflora*, namely, 2 pentamers, a trimer, and a dimer. These compounds have been named hopeasides A-D (Fig.24). The two pentamers are the first C-glucopyranosyl resveratrol oligomers isolated to date. Moreover, they are stereoisomers of each other but the orientation in which the two differ remains unknown. The orientation, interestingly, is in regard to the orientation of the C–C bonds between 7e-8e-9e. Theoretically, these are sp^3 hybridized freely rotatable bonds, however due to the steric hindrance within the molecule, rotation is restricted creating the two stereoisomers.

Figure 24. Hopeasides A-D (left to right) isolated from *Hopea parviflora*.

3. Biological activity of stilbenoids

Historically, there has been significant work done on the role of stilbenes and their activity as anti-bacterial agents, antioxidants, several anticancer properties, NF κ B and hemeoxygenase moderators. For example, kobophenol-A and -B are tetrastilbenes that were first isolated from *Carex kobomugi* and *Carex pumila*, respectively. These compounds have been shown to have moderate inhibitory activity against *Staphylococcus aureus* [3, 4]. Due to the inherent (poly)phenolic structure of stilbenes, there has been extensive research conducted on their role as antioxidants. Reactive oxygen species (ROS) are generated in bio-organic redox processes. Deregulation of this dynamic biological process leads to oxidative stress, which has been linked to many chronic human diseases including cancer, diabetes, and cardiovascular diseases [5]. The direct efficacy of stilbenes ability for the scavenging of ROS, or induce NADPH oxidase and xanthine oxidase inhibition, however still remains unclear [1].

An additional biological property that stilbenes exhibit is the inhibition of topoisomerase II. Topoisomerases play a critical role in the unwinding of coiled DNA during cellular transcription [6]. Upon discovering this mechanism for oligomeric stilbenes, Yamada et al. [6] conducted a study on over 40 stilbenes and their ability to inhibit topoisomerase II and identified α -viniferin as being highly active. In addition to its ability to inhibit topoisomerase II, α -viniferin has also been reported to not induce apoptosis, but interestingly arresting cell-cycle in the S-phase in human colon tumorigenic cells [7].

Resveratrol has also been implicated in the modulation of several proteins involved in a variety of degenerative diseases. It has been shown that resveratrol down regulates NF κ B, an important protein complex involved in cell survival and proliferation. Incorrect regulation of NF κ B has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development [8, 9]. Resveratrol has also been linked to moderate hemeoxygenase activity, which catalyzes the cleavage of heme to form iron, CO and bilirubin. Incorrect activity within hemeoxygenase has been linked to Parkinson's and Alzheimer's diseases [8, 9]. Finally, resveratrol has been shown to ameliorate some of the problems associated with type II diabetes, such as myocardial ischemia [40].

More recently, resveratrol and some of its analogs, have been linked to anti-aging properties. It was shown that through mimicking caloric restriction, resveratrol prolonged the lifespan of budding yeast, *Saccharomyces cerevisiae*. Supporting results

followed with additional organisms, including the nematode, *Caenorhabditis elegans*, the fruit fly, *Drosophila melanogaster*, and the honey bee, *Apis mellifera* [41–44]. With mammals, the first experiments carried out in mice confirmed that resveratrol mimicked the effects of caloric restriction including reduced albuminuria, decreased inflammation, and apoptosis in the vascular endothelium, increased aortic elasticity, greater motor coordination, reduced cataract formation, and preserved bone mineral density [45].

Since aging is a complex process, one could anticipate that the role that stilbenes play in exerting anti-aging effects could be achieved by targeting multiple physiological processes. In fact, resveratrol appears to fit such a hypothesis, although its exact mechanism is not yet fully established. The most intriguing observation linking resveratrol with longevity was its ability to activate members of the sirtuin (SIRT) family, especially SIRT1 [41]. SIRT1 has been shown to mediate the beneficial effects of caloric restriction on longevity extension [46]. Resveratrol was also shown to reverse a variety of age-related conditions by counteracting mitochondrial dysfunction and metabolic diseases [47]. Several of these effects are SIRT1-dependent, but many others are mediated through independent pathways, such as a cAMP-PKA-AMPKA cascade [48].

4. Summary and concluding remarks

In summary, this review covers the chemical structures of natural stilbenoids isolated and identified from 2009–2013. It is apparent that stilbenes are an exciting chemical

class of natural polyphenolic compounds with a unique carbon backbone that allows them to polymerize into complex structures and to be biologically active in a variety of systems. They have been implicated in the inhibition of growth of microbes, as well as to be strong anti-oxidants. In addition, this class of compounds has the ability to inhibit the growth of cancer cells in vitro. These compounds display great potential in their chemical diversity but understanding of their role in human health prevention and disease risk reduction would need further studies into their in vivo biological potential and mechanisms of action.

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MANUSCRIPT 2

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Phenolic Constituents of *Carex vulpinoidea* Seeds and their Tyrosinase Inhibitory

Activities. Niesen, Daniel B.; Ma, Hang; Yuan, Tao; Bach, Alvin C. II; Henry,

Geneive E.; Seeram, Navindra P. (2015). *Natural Product Communications*. 10, 491-

493.

Abstract

Two new phenolics, a stilbenoid, vulpinoideol A (1), and a chalcone, vulpinoideol B (2), along with ten known compounds (3-12) were isolated from *Carex vulpinoidea* seeds. The structures of compounds 1-12 were elucidated based on spectrometric and spectroscopic analyses including HRESIMS, 1D and 2D NMR data. All compounds were evaluated for their tyrosinase enzyme inhibitory activities.

1. Introduction

The *Carex* genus (family, Cyperaceae) contains over 2000 species [1], but the majority of these remain largely uninvestigated for their phytochemical constituents. This is unfortunate, considering that the Cyperaceae family is one of the few plant families known to produce stilbenoid derivatives, a sub-class of phenolic compounds which exhibit significant structural diversity and a wide range of biological activities [2, 3]. Over the past few years, our group has studied *Carex* species found in the northern regions of the United States, which have yielded several bioactive phenolic constituents, including resveratrol oligomers and other stilbenoids [4, 5].

Hyperpigmentary disorders, such as melasma and freckles, are due to the abnormal accumulation of melanin. The tyrosinase enzyme has been implicated as a key enzyme in melanogenesis, as it initiates an enzymatic cascade through the conversion of tyrosine and 3, 4-dihydroxyphenylalanine (DOPA) into DOPA-quinone [6, 7]. Notably, plant natural products, in particular, phenolic compounds, have shown great promise as tyrosinase inhibitors, a primary example being the phenolic glycoside, arbutin, which is widely used for commercial cosmetic applications [8, 9]. Moreover, several phenolic sub-classes, including flavonoids, chalcones, and stilbenoids, are known to be tyrosinase inhibitors [10-12]. Therefore, in the current study, we sought to: 1) isolate and chemically characterize the constituents from the seeds of the previously uninvestigated *Carex vulpinoidea* and, 2) evaluate the isolates for their anti-tyrosinase activities.

Herein we report the isolation and structure elucidation of two new phenolics, namely, vulpinoideols A (1) and B (2) (Figure 1), along with ten known compounds (3-12) (Figure 2) from *C. vulpinoidea* seeds. All of the isolates were evaluated for their tyrosinase inhibitory activity, along with the positive control, arbutin.

Compound 1 had a molecular formula of $C_{25}H_{30}O_8$ from the HRESIMS data corresponding to the quasimolecular ion at m/z 481.1835 $[M + Na]^+$ (Figure 3). The 1H NMR spectrum (Figure 4) revealed a *para*-substituted aromatic ring system (ring A) with *ortho*-coupled- protons at δH 7.34 (2H, $J = 8.5\text{Hz}$) and δH 6.78 (2H, $J = 8.5\text{Hz}$); a second aromatic spin system (ring B) with *meta*-coupled protons at δH 6.74 (1H, $J = 2.1\text{Hz}$) and δH 6.60 (1H, $J = 2.1\text{Hz}$); a *trans*-ethylene system with protons at δH 6.86 (1H, $J = 16.3\text{Hz}$) and δH 7.14 (1H, $J = 16.3\text{Hz}$); an olefinic proton at δH 5.14 (1H, $J = 6.4\text{Hz}$); one methylene, and two methyl groups and characteristic signals of a β -glucopyranose moiety, for which the resonance of the anomeric proton was at δH 4.86 ($J = 7.1\text{Hz}$).

The ^{13}C NMR data (Figure 5) coupled with the HSQC spectra (Figure 6) revealed the presence of 25 carbons, and confirmed the presence of two aromatic rings, containing three oxygen-linked carbons, two alkene double bonds, and a sugar moiety. Detailed analysis of the 2D NMR data (including HSQC and HMBC) (Figures 6 and 7) allowed the structure elucidation of compound 1 (Figure 1). The data revealed the presence of an isoprene group, which was confirmed through the following HMBC correlations, H-18/H-19 with C-16/C-17 and H-15 with C-16/C-17.

The placement of the isoprene group at position C-14 was based on HMBC correlations of H-15 with C-9/C-13/C-14 (Figure 1). The β -glucopyranose moiety was placed at position C-13 based on the HMBC correlation of the anomeric proton H-1a to C-13. Further HMBC correlations, including H-12 with C-10/C-11, and H-10 with C-12/C-13/C-14, allowed for the arrangement of substituents in ring B. Additional key HMBC correlations, H-7 to C-1/C8 and H-8 to C-7/C-10/C-14 indicated the presence of a C6-C2-C6, 1, 2-diphenylethylene moiety. This backbone is characteristic of a monomeric stilbene derivative and, therefore, compound 1 was elucidated as depicted and assigned the common name of vulpinoideol A.

Compound 2 (vulpinoideol B) was identified with a molecular formula of $C_{26}H_{32}O_9$, corresponding to a quasimolecular ion peak at m/z 511.1937 $[M + Na]^+$ (Figure 9). Compared with compound 1, vulpinoideol B (2) had the addition of a carbonyl carbon (C-8a, 206.2Hz), as well as the saturation of the alkene linkage between the aromatic spin systems, δH 2.90 (2H, t) and δH 3.16 (2H, t) (Figure 10). These differences suggested the backbone of a saturated chalcone. Similar to vulpinoideol A (1), vulpinoideol B (2) retained the *ortho*-coupled aromatic protons (δH 7.04, 2H, $J = 8.2$ Hz and δH 6.69, 2H, $J = 8.2$ Hz). However, its second aromatic system contained two isolated protons at δH 6.62 (1H, s) and δH 7.72 (1H, s) (Figure 11).

The ^{13}C NMR (Figure 12) and HMBC data (Figure 13) were again indicative of an isoprene substituent, as well as a β -glucopyranose moiety, which had its anomeric proton at δH 5.02 ($J = 6.9$ Hz). The placement of the isoprene unit at C-13 was

confirmed using similar HMBC correlations as vulpinodeol A (1) i.e. H-15 with C-14/C-13/C-12. Additionally, the HMBC correlation between the anomeric proton of the sugar, H-1a and C-12 allowed for the placement of the sugar moiety at C-12. The HMBC correlations H-14 and H-8 with C-8a, and H-7 with C-8/C-5 confirmed the structure of the backbone (Figure 1).

The inhibitory effects of all the isolates on mushroom tyrosinase enzyme were evaluated along with arbutin, the positive control. Among the isolates, only the new compound, vulpinoideol B (2), as well as the known compounds, hopeaphenol (4) and the α -hydroxychalone (8) were more active than arbutin (IC₅₀ values of 49.4, 6.1, 29.1 vs 72.6 μ M, respectively). The IC₅₀ values of all the other isolates, including the new compound, vulpinoideol A (1) (IC₅₀ = 151 μ M), exceeded 100 μ M.

2. Experimental

General: Semi-preparative HPLC separations were performed on a Hitachi Elite LaChrom system consisting of an L2130 pump, an L-2200 autosampler, and an L-2455 diode array detector, all operated by EZChrom Elite software, using a Phenomenex C18 column (250 × 10mm, 5μm), with a flow rate of 3mL/min. Medium pressure liquid chromatography (MPLC) separations were carried out on a prepacked C18 column (37 × 5.5cm; flow rate 3mL/min), and a liquid chromatography DLC-10/11 Isocratic pump (D-Star Instruments, Manassas, VA, USA). All solvents were either ACS or HPLC grade and were obtained from Wilkem Scientific (Pawcatuck, RI, USA). Sephadex LH20 resin (Amersham Biosciences) was packed in a glass column (3 × 70cm) and eluted with a 100% methanol mobile phase. HRESIMS data was acquired using a Synapt G2-S QTOF mass spectrometer (Waters, Milford, MA).

Plant material: The seeds of *Carex vulpinoidea* were collected on July 22, 2010 at the Susquehanna University CEER (Center for Environmental Education and Research) property in Pennsylvania, USA. A voucher sample (CM# 507015) is deposited at the Carnegie Museum Herbarium in Pittsburg (PA, USA).

Extraction and separation: The seeds were ground (39.6g) and exhaustively extracted sequentially with hexanes, acetone, and methanol (each 3 times, 150mL per solvent for 24h, each extraction). The acetone extract (2.03g) was subjected to C18 reverse phase MPLC with a gradient solvent system of MeOH: H₂O (40:60-100:0 v/v) to afford fractions 1-8. Fraction 1 (575.3mg) was eluted through the Sephadex LH20 column to

afford fractions 9-15. Fraction 12 (426.6mg) was subjected to reverse phase C18 MPLC (gradient, MeOH:H₂O, 30:70-50:50, v/v) to yield hopeaphenol (4, 18.6mg) [13] and grandiphenol A (5, 24.1mg) [14].

Fraction 5 (19.9mg) was eluted through the Sephadex LH20 column to afford sub-fractions 16-28. Two compounds, 3,5,5',7'-tetrahydroxyflavone (6, 3.4mg) [15] and a benzofuran (3, 2.1mg) [16], were isolated from fraction 22 (30.4mg) using semi-preparative reverse phase C18 HPLC (isocratic, 55:45 MeOH:H₂O, v/v). Similarly, fraction 6 (384.7mg) was chromatographed on the Sephadex LH20 column to afford sub-fractions 29-35, and further separation of sub-fraction 30 (50.9mg) by C18 HPLC (gradient, MeOH:H₂O, 50:50-100:0, v/v) yielded 3',4',7-trihydroxyflavone (7, 2.7mg) [17], vulpinoideol A (1, 3.4mg) and α -hydroxychalone (8, 4.4mg) [18]. Butein (9, 15.1mg) [19] was isolated from sub-fraction 32 by C18 HPLC (isocratic, MeOH:H₂O, 77:23, v/v). Methylated naringenin (10, 1.8mg) [20], vulpinoideol B (2, 2.2mg), and luteolin (11, 4.8mg) [5] were obtained from fraction 6 by C18 HPLC (gradient, MeOH:H₂O, 60:40 – 70:30, v/v). Bavachalcone (12, 1.3mg) [21] was isolated from fraction 9 using Sephadex LH20, followed by C18 HPLC purification (isocratic, MeOH:H₂O, 70:30, v/v).

Vulpinoideol A (1)

White amorphous solid.

UV (MeOH) λ_{max} : 305, 210 nm.

¹H NMR and ¹³C NMR: Table 1.

HRESIMS: m/z [M + Na]⁺ calc. for C₂₅H₃₀O₈: 458.5009; Found: 481.1835.

Vulpinoideol B (2)

Light yellow amorphous solid.

UV (MeOH) λ_{max} : 330, 275, 210 nm.

¹H NMR and ¹³C NMR: Table 1.

HRESIMS: m/z [M + Na]⁺ calc. for C₂₆H₃₂O₉: 488.5269; Found: 511.1937.

Tyrosinase inhibition assay: The tyrosinase assay was carried out similarly to Zhang and co-workers [22]. Briefly, mushroom tyrosinase, L-tyrosine and the positive control, 4-hydroxyphenyl β-D-glucopyranoside (arbutin) were purchased from Sigma-Aldrich (St. Louis, MO). Tyrosinase inhibition assays were performed in 96-well microplate format using a SpectraMax M2 microplate reader (Molecular Devices, CA). Test samples were dissolved in DMSO at a concentration of 2.0mM and then diluted to different concentrations with phosphate buffer (0.1M, pH 6.8). Each well contained 40μL of sample with 80μL of phosphate buffer solution, 40μL of tyrosinase (100units/mL), and 40μL L-tyrosine (2.5mM). The mixture was incubated for 30min at 37°C and absorbance was measured at 490nm. Each sample was accompanied by a blank, containing all components except L-tyrosine. Arbutin was used as the positive control. The results were compared with a negative control consisting of 10% DMSO in place of the sample. The percentage of tyrosinase inhibition was calculated as follows:

$$[(\Delta A_{\text{control}} - \Delta A_{\text{sample}}) / \Delta A_{\text{control}}] \times 100$$

3. Acknowledgments

The plant material was collected by Kristen Brown (currently at University of Maine) and authenticated by Bonnie Isaac of the Carnegie Museum Herbarium. We would like to thank Dr. Ke Liu from the University of Pittsburgh for assistance with the mass spectral data.

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Table 1 ¹H NMR and ¹³C NMR data of Vulpinoideols A and B.

No.	Vulpinoideol A		Vulpinoideol B	
	δ _C	δ _H (mult, <i>J</i> in Hz)	δ _C	δ _H (mult, <i>J</i> in Hz)
1	127.3	7.34 (2H, d, 8.5)	130.3	7.04 (2H, d, 8.2)
2	115.1	6.78 (2H, d, 8.5)	116.1	6.69 (2H, d, 8.2)
3	157.0	-	156.8	-
4	115.1	6.78 (2H, d, 8.5)	116.1	6.69 (2H, d, 8.6)
5	127.3	7.34 (2H, d, 8.5)	130.3	7.04 (2H, d, 8.2)
6	129.3	-	133.0	-
7	129.6	6.86 (d, 16.3)	31.3	2.90 (2H, t)
8	123.4	7.14 (d, 16.3)	41.5	3.16 (2H, t)
8a	**	**	206.2	-
9	138.4	-	115.1	-
10	105.5	6.74 (d, 2.1)	164.3	-
11	155.7	-	103.3	6.62 (s)
12	101.9	6.60 (d, 2.1)	162.9	-
13	156.3	-	123.5	-
14	120.0	-	131.8	7.72 (s)
15	24.0	3.40/3.56 (m)	28.5	3.28 (m)
16	124.4	5.14 (t, <i>J</i> =6.4)	123.4	5.31 (t)
17	129.4	-	133.9	-
18	16.8	1.83 (3H, s)	17.9	1.71 (3H, s)
19	24.5	1.68 (3H, s)	25.9	1.75 (3H, s)
1a	101.5	4.86 (7.1)	101.3	5.02 (6.9)
2a	73.5	3.50 (m)	78.3	3.51
3a	76.7	3.44 (m)	74.8	3.48
4a	69.8	3.44 (m)	71.2	3.44
5a	76.9	3.49 (m)	78.3	3.51
6a	61.1	3.74/3.94 (dd)	62.3	3.71, 3.90

Table 2: Tyrosinase Enzymatic Inhibition Activity of two new compounds, Vulpinoideols A and B, as well as other highlighted *C. vulpinodea* isolates. Arbutin (*) has been used a positive control.

Compounds	IC ₅₀ (μM)	Std dev.
Vulpinoideol A (1)	150	6.0
Vulpinoideol B (2)	49.4	13
Hopeaphenol (4)	6.10	1.2
Grandiphenol A(5)	174	5.8
α-hydroxychalcone (8)	29.1	0.3
Bavachalcone (12)	122	7.9
Arbutin*	72.6	7.9

Figure 1: Selected HMBC correlations (H→C) of vulpinoideol A (**1**) and vulpinoideol B (**2**) (top to bottom, respectively).

Figure 2: Known isolates from *C. vulpinoidea*

Figure 3. Vulpinoideol A. High Resolution Mass Spectrometry Data.

Figure 4. Vulpinoideol A - ^1H NMR spectrum. Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

Figure 5. Vulpinoideol A- ^{13}C NMR spectrum. Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

Figure
6. Vulpinoideol A - NMR HSQC spectrum. Data was measured in CH₃OD at 500 MHz (¹H) and 125 MHz (¹³C).

Figure 7. Vulpinoideol A - NMR HMBC spectrum. Data was measured in CH₃OD at 500 MHz (¹H) and 125 MHz (¹³C).

Figure 8. Vulpinoideol A - NMR COSY spectrum. Data was measured in CH₃OD at 500 MHz (¹H) and 125 MHz (¹³C).

Figure 9. Vulpinoideol B. High Resolution Mass Spectrometry Data.

Figure 10 Vulpinoideol B – ^1H NMR spectrum. Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

Figure 11. Vulpinoideol B - NMR HSQC spectrum. Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

Figure 12 Vulpinoideol B - ^{13}C NMR spectrum. Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

Figure 13. Vulpinoideol B - NMR HMBC spectrum. Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

Figure 14. Vulpinoideol B - NMR COSY spectrum. Data was measured in CH₃OD at 500 MHz (¹H) and 125 MHz (¹³C).

CHAPTER 2

GALLOTANNINS

Manuscript 3 appears as published:

Structure Activity Related, Mechanistic, and Modeling Studies of Gallotannins containing a Glucitol-Core and α -Glucosidase. Ma, Hang; Wang, Ling; Niesen, Daniel B.; Cai, Ang; Cho, Bongsup P.; Tan, Wen; Gu, Qiong; Xu, Jun; and Seeram, Navindra P. (2015). <i>RSC Advances</i> . 5, 107904-107915.....	70
Synthesis of tetragalloylglucitol (maplexin J).....	108

ABSTRACT

The incidence of type 2 diabetes mellitus is increasing rapidly worldwide and has become a significant public health burden [1]. Among various strategies used for type 2 diabetes management, clinical α -glucosidase inhibitory drugs, such as acarbose, are utilized to inhibit the activity of carbohydrate hydrolyzing enzymes and thus decreasing postprandial hyperglycemia [2]. Over the past decade, there has been renewed scientific interest in identifying plant-based α -glucosidase inhibitors for the treatment and management of diabetes. Moreover, since these purified natural products could serve as important leads in the development of new classes of glucosidase inhibitors, the synthesis of analogs have also attracted a considerable amount of scientific interest [3].

Our laboratory previously isolated a series of α -glucosidase inhibitory gallotannins, named maplexins A-I, from the red maple (*Acer rubrum*) species [4, 5]. These compounds contained mono-, di-, and tri-galloyl substituents located at different positions on a 1, 5-anhydro-*D*-glucitol moiety. Interestingly, the α -glucosidase inhibitory activities of these maplexins increased with the number of galloyl substituents, and a trigalloyl substituted maplexin was 20-fold more potent than the clinical drug, acarbose [5]. However, since a tetra-galloyl substituted maplexin was not obtained in our previous natural product isolation studies, a total synthesis is required to further evaluate the class of compounds and its α -glucosidase inhibitory activity.

To investigate the ligand-enzyme interaction, several biophysical methods including binding domains, modifications of hydrophobic surfaces, and secondary structural conformational changes were explored. Enzymatic kinetics and biophysical tools, including isothermal titration calorimetry (ITC), fluorescence, and circular dichroism (CD) spectroscopic methods, were used to elucidate the inhibitory mechanisms of the gallotannins against the α -glucosidase enzyme. Computational manipulation of homology modeling and molecular docking were also performed to support the prediction of binding interactions between the gallotannins and α -glucosidase.

With a synthetic route established for maplexins E and J, through a collaborative publication with Dr. Hang Ma (Manuscript 3), we were able to continue the investigation of the ligand-enzyme interaction of the class of gallotannins and their target enzyme α -glucosidase. As a collaborative publication, Dr. Ma's contribution specifically, sections: 2.2 measurements of α -glucosidase inhibitory assay, 2.3 kinetics of α -glucosidase inhibition, 2.5 hydrophobic interactions of α -glucosidase using bis-ANS, 2.6 circular dichroism (CD) spectroscopy, table 1, table 3, figure 2, figure 4, and the resultant analysis of that data found in sections: 2.2, 2.3, 2.5, 2.6, table 1, table 3, figure 2,, appear in Dr. Ma's dissertation (Dr. Hang Ma Dissertation, Phytochemical and biological investigation of gallotannins from red maple (*Acer rubrum*) species, University of Rhode Island, 2014).

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MANUSCRIPT 3

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Structure Activity Related, Mechanistic, and Modeling Studies of Gallotannins containing a Glucitol-Core and α -Glucosidase. Ma, Hang; Wang, Ling; Niesen, Daniel B.; Cai, Ang; Cho, Bongsup P.; Tan, Wen; Gu, Qiong; Xu, Jun; and Seeram, Navindra P. (2015). *RSC Advances*. 5, 107904-107915.

Abstract

Gallotannins containing a glucitol core, which are only produced by members of the maple (*Acer*) genus, are more potent α -glucosidase inhibitors than the clinical drug, acarbose. While this activity is influenced by the number of substituents on the glucitol core (e.g. more galloyl groups leads to increased activity), the mechanisms of inhibitory action are not known. Herein, we investigated ligand-enzyme interactions and binding mechanisms of a series of 'glucitol-core containing gallotannins (GCGs)' against the α -glucosidase enzyme. The GCGs included ginnalins A, B and C (containing two, one, and one galloyl/s, respectively), maplexin F (containing 3 galloyls) and maplexin J (containing 4 galloyls). All of the GCGs were noncompetitive inhibitors of α -glucosidase and their interactions with the enzyme were further explored using biophysical and spectroscopic measurements. Thermodynamic parameters (by isothermal titration calorimetry) revealed a 1:1 binding ratio between GCGs and α -glucosidase. The binding regions between the GCGs and α -glucosidase, probed by a fluorescent tag, 1,1'-bis(4-anilino-5-naphthalenesulfonic acid), revealed that the GCGs decreased the hydrophobic surface of the enzyme. In addition, circular dichroism analyses showed that the GCGs bind to α -glucosidase and lead to loss of the secondary α -helix structure of the protein. Also, molecular modeling was used to predict the binding site between the GCGs and the α -glucosidase enzyme. This is the first study to evaluate the mechanisms of inhibitory activities of gallotannins containing a glucitol core on α -glucosidase.

1. Introduction

In mammals, enzymes involved in the carbohydrate metabolic pathways have been the therapeutic targets for various diseases, including diabetes, cancer, and viral infections [1-3]. Among these enzymes, the group of glucosidases (EC 3.2.1.20) plays a crucial role in carbohydrate metabolism by catalyzing the cleavage of glycosidic linkage of oligosaccharides or glycoconjugates to release monosaccharides [4]. Inhibition of glucosidase hinders glucose absorption and consequently decreases postprandial blood glucose levels. Development of α -glucosidase inhibitors, as a class of antidiabetic medications for type II diabetes mellitus (T2DM), has attracted significant scientific attention [5]. In fact, several synthetic α -glucosidase inhibitors (AGIs), including acarbose, miglitol, and voglibose, are widely used as clinical treatment options for T2DM [6, 7]. However, adherence to synthetic AGI, as prescription medications, has suffered due to prominent undesirable side effects, including flatulence, diarrhea, and abdominal pain [8]. Conversely, naturally occurring AGIs, isolated from medicinal plants and medicinal foods, offer an attractive dietary strategy for T2DM management since they tend to be safe, and have fewer adverse effects than synthetic AGIs [9].

The maple (*Acer*) genus is widely regarded for certain species including the sugar maple (*Acer saccharum*) and red maple (*Acer rubrum*) species, the sap of which are collected and concentrated to produce the natural sweetener, maple syrup [10]. Interestingly, in the plant kingdom, only members of the maple (*Acer*) genus are

reported to produce gallotannins containing a glucitol instead of a glucose core which is most commonly found in gallotannins [11-16].

Our group and others have shown that these ‘glucitol-core containing gallotannins (GCGs)’ display potent α -glucosidase inhibitory properties which is influenced by the number, type, and location of substituents on their glucitol core [14-17]. In addition, our group showed that a red maple bark extract, enriched in GCGs, was similar in activity as acarbose, in lowering blood glucose in an animal model [18]. Therefore, GCGs represent an interesting polyphenol sub-class of plant natural products which show great promise as natural AGIs.

In our group’s research efforts to identify natural AGIs, we reported on a series of new GCGs, named maplexins E-I, from the red maple (*Acer rubrum*) species [14-16]. The GCGs feature different numbers and positions of galloyl groups attached to a 1,5-anhydro-D-glucitol core and exhibited 20-fold higher potency than acarbose which increased with the number of galloyl groups on the glucitol moiety. However, since these naturally occurring/isolated GCGs only contained three galloyl groups at the most, in an effort to broaden our test panel of GCGs, we synthesized a tetragalloylglucitol analog assigned the common name of maplexin J. Indeed, maplexin J was found to be three times more potent than maplexins E and F (contain trigalloyls), and 70 times more potent than the competitive inhibitor acarbose (see Table 1), confirming our previous SAR observations. In the current study, we have focused our attention on investigating the mechanisms of inhibitory actions of these

GCGs on the α -glucosidase enzyme. This research project was also prompted, in part, by the fact that other natural AGIs, such as 1-deoxynojirimycin, resveratrol, and oxyresveratrol, show different mechanisms of action against α -glucosidase [20-21].

The mechanisms of inhibition of compounds on the α -glucosidase enzyme can be investigated by several biophysical methods including binding domains, modifications of hydrophobic surfaces, and secondary structural conformational changes of enzymes induced by binding ligands [21]. In this study, enzymatic kinetics and biophysical tools, including isothermal titration calorimetry (ITC), fluorescence, and circular dichroism (CD) spectroscopic methods, were used to elucidate the inhibitory mechanisms of the GCGs against the α -glucosidase enzyme. Computational manipulation of homology modeling and molecular docking were performed to support the prediction of binding actions between the GCGs and the α glucosidase enzyme.

2. Experimental

2.1 Chemicals.

α -Glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae*, p-nitrophenyl- α -D-glucopyranoside (pNPG), and bis-8-anilinonaphthalene-1-sulfonate (bis-ANS) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals were analytical reagent grade and were purchased from Sigma Aldrich, unless otherwise specified. The naturally occurring GCGs (Figure 1): ginnalin A (GA; contains two galloyls), ginnalin B (GB; contains one galloyl), ginnalin C (GC; contains one galloyl) and maplexin F (MF; contains three galloyls) were isolated from the red maple species as previously reported [14, 15].

2.2 Measurements of α -glucosidase inhibitory assay.

A mixture of 50 μ L test samples and 100 μ L 0.1M phosphate buffer (pH 6.9) containing yeast α -glucosidase solution (1.0 U/mL) were incubated in 96-well plates at 25°C for 10minutes. After pre-incubation, 50 μ L of 5mM pnitrophenyl- α -D-glucopyranoside solution in 0.1M phosphate buffer (pH 6.9) were added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 minutes. Before and after incubation, absorbance was recorded at 405nm by a micro-plate reader (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA) and compared to that of the control which had 50 μ L buffer solutions in place of the samples. The α -glucosidase inhibitory activity was expressed as inhibition % and was calculated as: Inhibition % = $100 \times [(C5 - C0) - (S0 - S5)] / (C5 - C0)$, where C0 is the absorbance of the reagent blank in 0 minute,

S0 the absorbance of the samples in 0 minute, C5 the absorbance of the reagent blank in 5 minutes, and S5 the absorbance of the samples in 5 minutes [22].

2.3 Kinetics of α -glucosidase inhibition.

The inhibition types of ginnalin A (GA), maplexin F (MF), and maplexins J (MJ) were determined from Lineweaver–Burk plots, using previously reported methods with minor modifications [23]. Typically, two concentrations around the IC₅₀ values of each sample were chosen (ranging from 2 – 250 μ M). For each concentration, α -glucosidase activities were tested by using different concentrations of pPNP glycoside (1 to 1000 μ M). The mixtures of the enzyme and the inhibitor were dissolved in 50mM phosphate buffer (pH 6.8), and pre-incubated at room temperature for 30 minutes, and then the substrate was added. The enzymatic reaction was carried out at room temperature for 60 seconds, and monitored spectrophotometrically by measuring the absorbance at 405nm. Inhibition types of the inhibitors were determined by double-reciprocal plots.

2.4 Titration microcalorimetry.

Isothermal titration calorimetry (ITC), used to determine the thermodynamic properties between the GCGs and α -glucosidase, were measured with a VP-ITC Microcalorimeter (MicroCal, Northampton, MA, USA) according to previously reported methods with modifications [24]. A typical titration experiment consisted of 17 consecutive injections at 240s intervals consisting of 14 μ L injections of each GCGs into the titration cell at 25°C in phosphate buffer (pH 7.0, 100mM). The

titration cell was stirred continuously at 310 rpm. The GCGs (1-4mM) and α -glucosidase enzyme (0.026-0.031mM) were dissolved in the same phosphate buffer and degassed for 20 mins under vacuum using a ThermoVac (MicroCal, Northampton, MA, USA) prior to each experiment. Controls included buffer injected into buffer and GCGs injected into buffer solutions, respectively. Data was analyzed by using nonlinear regression with a single-site binding model in VP Viewer 2000, which uses the scientific plotting software, ORIGIN 7 (Origin Lab. Corp., Northampton, MA, USA).

2.5 Hydrophobic interactions of α -glucosidase using bis-ANS.

α -glucosidase (2 μ M) was incubated in the presence of various concentrations of the GCGs (0-80 μ M) at 37°C for 5 minutes. Bis-ANS (5 μ M) was then added, and fluorescence was measured after incubation at 37°C for 15 minutes ($\lambda_{\text{ex}} = 400\text{nm}$, $\lambda_{\text{em}} = 440\text{--}600\text{ nm}$) [25].

2.6 Circular dichroism (CD) spectroscopy.

Far UV CD measurements (190-240nm) were conducted on a Jasco J-810 spectropolarimeter equipped with a Peltier temperature controller at 25°C. Briefly, 2 μ M of α -glucosidase was treated with various concentrations of the GCGs (0-80 μ M). The samples were dissolved in 200 μ L of a sodium phosphate buffer (0.1M, pH 6.8) and placed in a 1.0mm path length cell. The spectra was collected and corrected by subtraction of a blank 0.1M sodium phosphate buffer (pH 6.8), reducing noise and smoothing [26]. The changes of secondary structure of α -glucosidase were

estimated according to a method in the DichroWeb program, an online server for protein secondary structure analyses from the CD spectroscopic data [27, 28].

2.7 Homology modeling of α -glucosidase.

The *Saccharomyces cerevisiae* α -glucosidase was downloaded from the UniProt protein knowledgebase (accession number P53341) [29]. The structure of isomaltase from *Saccharomyces cerevisiae* (PDB entry 3A4A) that shared a 71.8% sequence identity and 87.1% sequence similarity with *Saccharomyces cerevisiae* α -glucosidase based on National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) search results was selected as the template for homology modeling. Sequence alignment and structural model building were performed using DS 3.5 (Discovery Studio, version 3.5, Accelrys, Inc., San Diego, CA, USA). Ten initial 3D α -glucosidase structures were constructed and sorted in ascending order of PDF total energy score. The best structure with the most negative score was selected for further refinements.

2.8 Refinement of 3D α -glucosidase structure.

Molecular dynamics (MD) simulation is widely used for both homology and X-ray structural refinement [30, 31]. The initial best 3D α -glucosidase structure from homology modeling was refined through MD simulations using Amber12 [32]. The initial structure was solvated in an octahedron periodic box (based on the TIP3P model) with 12Å of water along each dimension. The protein was applied with the

Amber ff99SB force field and counter-ions were added to the system to neutralize charges [33].

MD simulations consisted of energy minimization, heat phase, equilibration, and production. The solvated system was minimized by three steps. First, a harmonic constraint potential of $10\text{kcal/mol/\AA}^2$ was applied to all atoms except water molecules. Second, the protein backbone atoms were restrained with a force of $5.0\text{kcal/mol/\AA}^2$. Finally, all atoms were allowed to move freely. In each step, energy minimization was executed by the steepest descent method for the first 2000 steps and the conjugated gradient method for the subsequent 2000 steps. After minimization, the system was gradually heated in the canonical “NVT ensemble” from 0 to 300 Kelvin (K) in 100 picoseconds (ps) using a Langevin thermostat with a coupling coefficient of 1.0ps with a force constant $10\text{kcal/mol/\AA}^2$ on the protein. The system was then equilibrated for the first two 500ps and subsequent three 200ps at 300K with decreasing restraint weights reduced from 10 to $0.2\text{kcal/mol/\AA}^2$. A production simulation run for 10ns was lastly performed at 300K. During the MD simulations, the long-range Coulombic interactions were handled using the particle mesh Ewald (PME) method and the cutoff distance for the long-range van der Waals (vdW) energy term was set at $10.0\text{\AA}$ [34]. All hydrogen atoms were constrained using the “SHAKE” algorithm and the time step was set at 2 femtosecond. Coordinate trajectories were recorded every 2ps. The refined 3D α -glucosidase structure was obtained from last snapshot of MD simulations and validated by Profiles-3D Verify module in DS 3.5.

2.9 Molecular docking.

GA, MF, and MJ were optimized using MMFF94s force field. The refined α -glucosidase structure from MD simulations was protonated based on amber99 force field after removing water molecules. To obtain the most plausible binding sites, we searched the whole 3-D space of the receptors, including both the active-site (Asp214, Glu276 and Asp349) and non-active-site regions. Site Finder module encoded in MOE (MOE 2010.10. Chemical Computing Group, Inc. Montreal, Canada) was employed for detecting potential ligand binding sites of α -glucosidase. MOE-docking was employed to identify the binding poses of GA, MF, MJ and α -glucosidase. During docking study, 30 poses per compound were retained. All docked poses of GA, MF, and MJ were ranked on the basis of the binding docking energies according to London G score [35, 36]. The best conformation for each compound from clustering results was chosen for binding mode analyses.

3. Results

3.1 Comparison of α -glucosidase inhibitory effects of GCGs

As shown in Table 1, the inhibitory activities of the GCGs against the α -glucosidase enzyme increased with increasing number of galloyl groups attached to the 1,5-anhydro-D-glucitol moiety. The mono-galloyl substituted gallotannins (GB and GC) did not demonstrate any inhibitory activities on α -glucosidase ($IC_{50} > 1000\mu M$), and the di-galloyl gallotannin (GA) only showed a moderate inhibitory activity with an IC_{50} value of $216.43\mu M$. However, the activities of MF and MJ, which contain three and four galloyl groups, respectively, were significantly enhanced ($IC_{50} = 13.70$ and $4.27\mu M$, respectively). The results indicated that MF and MJ were potent α -glucosidase inhibitors.

To further evaluate the inhibitory characteristics of GA, MF and MJ, enzyme kinetic assays were performed and their inhibition types were determined by the Lineweaver-Burk plots (Figure 3). The Lineweaver-Burk plot of each GCG (at different concentrations ranging from $2 - 250\mu M$) generated straight lines that all intersected the X-axis at the same point, suggesting that the GCGs were typical noncompetitive inhibitors of α -glucosidase. Therefore, this implied that GA, MF, and MJ bind to the noncompetitive site of the yeast's α -glucosidase enzyme rather than to the catalytic domain of the enzyme.

3.2 ITC measurement

The thermodynamic properties of the binding interaction between the GCGs and α -glucosidase complex were determined by using ITC. The titration peaks of GA, GB, and GC (at concentrations ranging from 1-4mM) were too weak to yield thermodynamic parameters. However, the titration peaks for MF or MJ (concentrations ranging from 1-2mM) showed binding interactions that were typically exothermic (see Figure 3). The thermodynamic parameters for GA, MF, and MJ, obtained from the ITC analyses, are summarized in Table 2. Both MF and MJ had a ligand:enzyme ratio (N value) of 1:1 indicating that one molecule of each of these GCGs binds to one molecule of the enzyme [37]. In addition, the binding constants (K_a) of MF or MJ with α -glucosidase were 2.85×10^4 and 4.22×10^4 mol⁻¹, respectively. The stronger binding affinity of MJ with α -glucosidase, compared to that of MF and the enzyme, was in agreement with the results obtained from the α -glucosidase inhibitory assay with these GCGs (Table 1) [38]. The negative ΔG values for both MF and MJ (-6.09 and -6.31kcal/mol, respectively) suggested that their binding interactions were spontaneous [38, 39]. Furthermore, a positive ΔS value and a negative ΔH value for both MF and MJ indicated that their binding interactions with the α -glucosidase enzyme were enthalpy-driven [39, 40]. Notably, the thermodynamic parameters observed for MF and MJ are similar to those reported for the major gallotannin present in tea, namely, epigallocatechin gallate (EGCG), with lipase [38].

Given that a positive ΔS value may be associated with the first stage of binding interaction, [41, 42] where the ligand and enzyme are immobilized in a hydrophobic environment, we further examined the hydrophobic alteration of the α -glucosidase enzyme.

3.3 GCGs reduced the hydrophobicity of α -glucosidase.

Noncovalent fluorescent probes have been extensively used for the study of protein conformation [43, 44]. An external fluorescent probe such as bis-8-anilinonaphthalene-1-sulfonate (bis-ANS) is sensitive to the protein microenvironment and selectively binds to the hydrophobic surface of protein [45]. Therefore, a bis-ANS probe was utilized to assess the exposure of α -glucosidase hydrophobic surface induced by the ligands. As shown in Figure 4, after being co-incubated with α -glucosidase for 20min at 37°C, GA, MF, and MJ were able to decrease the fluorescence of the bis-ANS-enzyme complex in a concentration-dependent manner. Although GA slightly decreased the fluorescent intensity of the bis-ANS-enzyme complex at the low concentration (20 μ M), compared to the non-treatment group, the fluorescence of the MF and MJ treatment groups were significantly reduced. Moreover, all three ligands greatly decreased the fluorescence at concentration of 80 μ M, suggesting that GA, MF, and MJ could decrease the hydrophobic surfaces of α -glucosidase.

Since hydrophobic surface is crucial to facilitate the formation of the enzyme active site, GA, MF, and MJ could have possibly inhibited the α -glucosidase enzyme activity by inducing poor hydrophobic surfaces on the enzyme structure [46].

3.4 Change in conformation of α -glucosidase induced by GCGs.

To further examine the effects of the GCGs on the secondary structure of α -glucosidase, circular dichroism (CD) spectra of free α -glucosidase and ligand- α -glucosidase complex were acquired. In Figure 5, the CD spectra of the free α -glucosidase enzyme exhibited two characteristic negative bands at 208 and 222nm, indicating that the major secondary protein structure presented in the α -glucosidase was α -helix [47, 48]. When various concentrations (20-80 μ M) of the GCGs were co-incubated with α -glucosidase, the CD spectra of the ligand-enzyme complex was significantly altered indicating that the conformation of the enzyme protein was affected by the ligands in a concentration dependent manner. As summarized in Table 3, the GCGs bind to the enzyme protein that mainly resulted in the loss of α -helix conformation. When 40 μ M of MF and MJ were delivered to α -glucosidase, the enzyme lost 4.9 and 5.6% of its α -helix conformation, respectively, which could potentially cause the loss of biological functions of the α -glucosidase enzyme.

3.5 Homology modeling.

Lacking the 3D structure of *Saccharomyces cerevisiae* α -glucosidase used in biological assays, we constructed a 3D homology structure of α -glucosidase by computational approach. The 3D homology models of α -glucosidase were generated

with the MODELER module in DS 3.5, and the best model with lowest PDF total energy score was refined by 10ns MD simulations (Figure 6B). Trajectory-based analysis showed that the root-mean-square deviation (RMSD) of the protein backbone atoms with reference to the initial structural coordinates increased slowly in the first 4 nanoseconds (ns) and then subsequently stabilized after 4 ns, suggesting that the α -glucosidase structure was stable.

The refined structure from the last snapshot of MD simulations was validated via Profile-3D, Procheck, and Prostat analysis. The overall self-compatibility score generated by Profile-3D module in DS 3.5 for this refined model was 252.5 (the verified expected high score was 263.47, and the verified expected low score was 118.56). Procheck was used to calculate the ϕ and ψ angles (Figure 6A) and four residues (Ser331, Glu522, Arg543, and Tyr372) were located in un-allowed regions, and the percentage of residues within the Ramachandran plot's allowed regions was 99.32%. Prostat analysis showed that almost all bond lengths, angles, and torsions stayed within a rational range. All of these validation results proved that the refined model is reliable.

3.6 Binding modes analyses.

A total of 35 potential ligand binding sites of the refined model were obtained from Site Finder module in MOE. The largest five binding sites (size>100) were selected for the docking study (Figure 6). As shown in Table 4, the three GCGs, namely, GA, MF, and MJ, shared the same scaffold, suggesting that they bind at the same site for

a given target. Sites 2, 4, and 5 were not suitable for these compounds because the trends of the docking scores for GA, MF, and MJ were inconsistent with the corresponding enzyme assay results. For example, compound MF in binding site 4 with a docking score of -17.37Kcal/mol is lower than that of compound MJ (-15.03Kcal/mol) which is inconsistent with their enzyme inhibition values (13.70 μ M for MF and 4.27 μ M for MJ). Similar trends were observed in the docking results of binding sites 2 and 5.

As shown in Figure 7C, binding site 3 was located at two helix structures and the three GCGs showed considerable binding modes based on the docking score and the enzyme inhibition assay. As previous studies have suggested that the helix structure could be stabilized by targeting compounds with polyhydroxyl groups, [21, 23, 49] our CD results suggest that the helix structures were reduced with increasing concentrations of GA, MF, and MJ. Therefore, this implied that binding site 3 is not targeted by these GCGs.

Binding site 1 is surrounded by coil, bend, and sheet structures, with little helix structures, which contain both active and inactive sites (Figure 7A). The trends of docking score of GA, MF, MJ, and the enzyme assay results are consistent. Based on all of the above analyses, the GCGs, GA, MF, and MJ target the binding site 1 of *Saccharomyces cerevisiae* α -glucosidase. The active site of α -glucosidase is comprised of Asp214, Glu276 and Asp349 and is represented by the red oval in Figure 8D. Superimposition of the binding modes of GA, MF, and MJ suggested

that all of the binding sites are located at the noncompetitive domain of α -glucosidase (Figure 8A, B, and C), which are relatively close to the active site (Figure 8D). All of the docking results demonstrate that GA, MF, and MJ are noncompetitive inhibitors, in accordance with the aforementioned kinetic results (Figure 2).

As shown in figure 8A, the hydroxyl group of GA can form two hydrogen bonds with the side chain of Ser244 and Phe157. One galloyl group of GA is inserted in the polar area comprised of Arg312, Glu304, Arg439, and Gln350, suggesting that a polar interaction can be formed. Moreover, the phenyl group can form favorable hydrophobic interactions with the side chains of Phe158, Phe157, His239, and His279. Compounds MF and MJ contain more galloyl groups than GA, suggesting that more favorable binding interactions will be formed against a given target according to Ge and coworkers' results [50]. As shown in figures 8B and 8C, MF can form four hydrogen bonds with Asp408, Glu304, Ser244, and Phe157, whereas MJ can form eight hydrogen bonds with Glu304, Ser244, Ser299, Glu276, Arg212, Thr215, and Ala216. Thus, hydrogen bonds are formed via the hydroxyl groups of these GCGs. Notably, a similar phenomenon has been observed in other phenolic compounds such as xanthone derivatives [23], resveratrol, and oxyresveratrol [21]. Moreover, more favorable hydrophobic interactions are formed between MF and MJ and α -glucosidase residues (Phe157, Phe158, Leu237, His239, His279, Phe300, and Ala278 for MF, and Phe157, Pro309, Leu237, His239, Leu218, His279, and Arg312 for MJ). These hydrophobic sites were occupied by GCGs, suggesting that the GCGs

decreased the hydrophobic surface of the enzyme which was consistent with the data obtained from the bis-ANS assay. Also, the binding energy of GA, MF, and MJ is -10.45, -13.02, and -16.35kcal/mol, respectively, which was in full accordance with results from the enzyme inhibition assay and binding model analyses.

4. Discussion

The red maple (*Acer rubrum*) species is endemic to eastern North America and widely known for its tree sap which is used to produce the natural sweetener, maple syrup. This plant has also been used by the Native Americans as an herbal medicine, and recent phytochemical and biological studies from our group on this species led to the discovery of several GCGs [14-16]. More importantly, these GCGs show potential as natural AGIs based on in vitro and animal studies [14-16, 18]. While several of these GCGs showed far more potent α -glucosidase inhibitory activities than the clinical drug, acarbose, their mechanisms of action remained unknown. Herein, we reveal several key mechanisms by which these GCGs interact with α -glucosidase.

First, the inhibition type of the GCGs against the α -glucosidase enzyme was found to be noncompetitive, suggesting that these gallotannins bind to the specific site of enzyme-substrate complex rather than the enzyme catalytic domain. This binding pattern is in agreement with previous studies of a 'glucose-core containing gallotannin', namely, pentagalloyl glucose (PGG), which is also a noncompetitive α -glucosidase inhibitor [51]. It is noteworthy that this distinguishes the GCGs from acarbose, a synthetic AGI, which is known to be a competitive inhibitor that directly binds to the active site of α -glucosidase [52]. In addition, increasing the number of galloyl groups on the glucitol core enhanced the α -glucosidase inhibitory activities of these GCGs, an SAR effect that has also been observed in glucose-core containing gallotannin α -glucosidase inhibitors, namely, PGG and its analogs [51].

Second, ITC analyses revealed that the binding stoichiometry between the GCGs (MF and MJ) and α -glucosidase was in a 1:1 ratio suggesting a single binding site for these compounds. This data is similar to that reported for the tea gallotannin, epigallocatechin gallate (EGCG) and lipase [38]. Additionally, the thermodynamic parameters (Table 2) revealed that the formation of the ligand-enzyme complex was spontaneous and enthalpy-driven suggesting that the binding interaction was largely attributed to the hydrogen bonding. This is not surprising since it has been well established that gallotannins form hydrogen bonds with the polar residues of proteins through their galloyl and/or hydroxyl groups [53, 54]. This observation was further supported by our computational models. Given that gallotannins-protein binding can be enthalpy-driven (by hydrogen bonding and protonation) [55-57], entropic factors (such as hydrophobic interactions) [41], are also often involved in the formation of ligand-protein complexes. Therefore, we examined the hydrophobicity of the GCGs and α -glucosidase complex.

The GCGs reduced the hydrophobic surface of the enzyme and impeded the formation of the active center of the α -glucosidase enzyme. GA, MF, and MJ significantly decreased the α -helix conformation of the enzyme protein. Since α -helix is a rigid secondary structure that maintains many enzymatic protein functions, loss of this structure may result in enzyme destabilization. Therefore, the major inhibitory mechanism of the GCGs against α -glucosidase mainly consists of binding

to the enzyme-substrate complex, reducing the hydrophobic surface, and changing the α -helix conformation of the enzyme protein.

Lastly, the GCGs act as noncompetitive inhibitors that bind to a noncompetitive domain of α -glucosidase located at relatively close to the active site (Figure 7D). This noncompetitive domain is different from the binding pocket of acarbose (competitive inhibitor) [52]. The predicted noncompetitive binding site may provide a novel site for structure-based discovering and designing novel α -glucosidase inhibitors. The molecular docking results demonstrate that more galloyl groups in the GCGs leads to enhanced binding affinity against α -glucosidase. However, further in silico studies to investigate the dynamic mechanisms of whether GCGs could trigger the loss of the secondary α -helix of the flexible structures (binding sites of GCGs) is warranted and included in our group's future studies.

Therefore, overall, this study provides useful information on these GCGs which can be used to guide the design of new GCG analogs with superior activities against α -glucosidase.

5. Conclusion

In summary, we have determined the inhibition type of ‘glucitol-core containing gallotannins (GCGs)’. The ligand-enzyme interaction was studied using several biophysical and spectroscopic tools including ITC, fluorescent probe and CD spectra. Our results showed that these GCGs had a single binding site, they could decrease the hydrophobic surfaces of the enzyme protein, and also reduce the α -helix conformation content of the protein’s secondary structure. Our study provides valuable information underlying the interactions between these GCGs and α -glucosidase, a crucial step towards further investigating their potential as natural AGIs. Further investigation of these GCGs will focus on in vivo studies to evaluate their safety and efficacy as potential dietary agents for T2DM management which is included in our group’s future planned studies.

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Table 1: α -Glucosidase inhibitory activities of GCGs

Compound	# of galloyl group	IC ₅₀ (μ M) ^a	Type of inhibition
ginnalin B (GB)	1	> 1000	n.d.
ginnalin C (GC)	1	> 1000	n.d.
ginnalin A (GA)	2	216.4 $\pm$ 3.19	noncompetitive
maplexin F (MF)	3	13.70 $\pm$ 0.87	noncompetitive
maplexin J (MJ)	4	4.270 $\pm$ 0.13	noncompetitive
acarbose ^b	—	142.4 $\pm$ 1.68	competitive

^a

IC₅₀ values are shown as mean $\pm$ S.D. from three independent experiments;

^b

Positive control; n.d. = not determined.

Table 2: Thermodynamic parameters for GCGs [ginnalin A (GA); maplexin F (MF) and maplexin J (MJ)] binding to α -glucosidase enzyme

Ligand	N [GCGs/ α -glucosidase]	K_d [10^4 mol^{-1}]	ΔG [kJ mol^{-1}]	ΔH [kJ mol^{-1}]	ΔS [J $\text{mol}^{-1} \text{ K}^{-1}$]
GA	n.d.	n.d.	n.d.	n.d.	n.d.
MF	1.01	2.85	-6.09	-10.26	14.06
MJ	1.01	4.22	-6.31	-25.17	63.31

n.d.: not detected

Table 3: The effect of GCGs (GA, MF, and MJ) on the secondary structure of α -glucosidase enzyme

Ligands (Conc.)	α -Helix %	β -Fold %	β -Turns %	Unordered
Control	36.3	21.9	14.3	26.9
GA 40 μ M	37.8	22.7	11.5	27.8
GA 80 μ M	33.5	22.5	15.8	27.7
MF 20 μ M	30.7	24.7	15.6	28.4
MF 40 μ M	31.2	22.7	16.6	28.9
MJ 20 μ M	31.8	21.1	20.4	26.4
MJ 40 μ M	30.7	24.4	19.1	25.3

Table 4: Detailed docking results for GA, MF, and MJ. Cluster represents the docking score of the lowest binding energy conformation of the more populated cluster.

compound		GA	MF	MJ
Site 1	Range	(-17.59, -8.53)	(-19.87, -11.98)	(-21.70, -9.40)
	cluster	-10.54	-12.02	-16.53
Site 2	Range	(-12.57, -5.78)	(-12.56, -6.80)	(-13.77, -6.39)
	cluster	-8.44	-8.15	-9.14
Site 3	Range	(-16.11, -6.47)	(-18.20, -8.70)	(-19.47, -7.70)
	cluster	-11.27	-12.25	-13.04
Site 4	Range	(-14.41, -5.28)	(-17.37, -6.43)	(-15.03, -5.90)
	cluster	-10.26	-10.37	-9.86
Site 5	Range	(-15.24, -7.46)	(-17.78, -6.23)	(-16.00, -7.53)
	cluster	-11.32	-11.72	-11.06

Figure 1: Chemical structures of five ‘glucitol core containing gallotannins (GCGs)’: ginnalin A, ginnalin B, ginnalin C, maplexin F and maplexin J.

Figure 2: Lineweaver-Burk plots of the kinetics of inhibition of GA (A), MF (B) and MJ (C) on α -glucosidase enzyme. Two concentrations (ranging from 2 - 250 μM of ligands; close to their IC_{50} values) were co-incubated with α -glucosidase at 37°C for 30min, then pNPG was added at varying concentrations (from 1-1000 μM).

Figure 3: Results of isothermal titration calorimetry (ITC) for GCGs binding to α -glucosidase: (Top) Raw data plot of heat flow against time for the titration of MF (A) or MJ (B) into 0.031mM α -glucosidase enzyme protein. (Bottom) Plot of molar enthalpy change against GCGs/ α glucosidase enzyme molar ratio.

Figure 4: Fluorescence intensity of bis-ANS- α -glucosidase complex. The changes of fluorescence intensity were induced by GA (A), MF (B) and MJ (C) at different concentrations ranging from 20 – 80 μ M.

Figure 5: Circular dichroism (CD) spectra of the α -glucosidase-GCG complex. α -glucosidase (2 μ M) were co-incubated with GA, MF, or MJ at 20-80 μ M at 37°C for 20 min.

Figure 6: The properties of the refined α -glucosidase structure. (A) Ramachandran plot for a 3D model of α -glucosidase (residues denoted with a + are outliers); (B) Time dependences of the root mean square deviations (RMSD) of the backbone atoms (C α , N, O-atoms) with respect to initial structure.

Figure 7: Top five potential ligand binding site generated via Site Finder module in MOE2010.10. The binding site is represented by alpha sphere centers and red oval. The active site is comprised of catalytic residues (Asp 214, Glu276, and Asp349).

Figure 8: Binding modes for GA (A), MF (B), MJ (C), and superimposition of the binding modes of GA (red), MF (aquamarine blue), and MJ (blue). Hydrogen bonds are depicted by red dotted lines. Red oval represents the active binding site.

Synthesis of tetragalloylglucitol (maplexin J)

The tetragalloylglucitol gallotannin (assigned the common name of maplexin J (MJ)) was synthesized by modification of the previously published method as shown in Scheme 1 [19]. Briefly, gallic acid (i, 101mg, 0.6mmol) was dissolved in dry *N,N*-dimethylformamide (DMF, 2mL). Imidazole (513mg, 7.5mmol) and *tert*-butyldimethylsilyl chloride (TBDMS, 521mg, 3.5mmol) were added to the solution and stirred at room temperature under nitrogen atmosphere for 24 hours. A white crystalline product precipitated out of the reaction solution. Trisilyl-protected gallic acid (ii) was isolated (244mg, 82%) from the precipitant using silica gel column chromatography.

Compound ii (187mg, 0.4mmol) and glucitol (iii, 10.4mg, 0.06mmol) were dissolved in dry dichloromethane (DCM, 2mL). *N, N'*-diisopropylcarbodiimide (DIC, 61.4mg, 0.5mmol) was added followed by 4-dimethylaminopyridine (DMAP, 74.4mg, 0.06mmol). The mixture was stirred at room temperature under nitrogen atmosphere for 96 hours. The crude product was purified using silica gel column chromatography to yield compound iv.

Compound iv (843mg, 64.6%) was isolated and its structure was confirmed by 2D NMR heteronuclear multiple bond correlations (HMBC) from the three sugar methines and the sugar methylene to the respective carbonyl carbons of the gallic acids. Deprotection of compound iv was accomplished in the presence of tetra-*n*-butylammonium fluoride (TBAF). Compound iv (50mg, 0.023mmol) was dissolved in dry tetrahydrofuran (THF, 2mL). TBAF (61.1mg, 0.23mmol) was added, and the mixture was stirred at room temperature under a nitrogen atmosphere for 10 minutes.

The crude product was purified using reverse-phase high-pressure liquid chromatography (HPLC) to yield the final product (10mg, 52.3%), which was characterized as tetragalloylglucitol and assigned the common name of maplexin J (MJ) based on its nuclear magnetic resonance (NMR) spectroscopic data (Table 1; key HMBC correlations shown in Figure 1). This scheme was carried out several times to synthesize enough maplexin J for further biological assays, fortunately maplexin E, a tri-galloylglucitol, was isolated and elucidated as an additional side product.

Scheme 1: Synthetic scheme of maplexin J.

Table 1. The ^1H and ^{13}C NMR data of maplexin J. Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

No.	δC	δH (mult, J in Hz)
Glucitol Sugar Core		
1	66.6	4.20 (m) 3.54 (t, 11.1, 12.2)
2	69.6	5.19 (ddd)
3	73.8	5.65 (t, 9.9, 11.1)
4	68.9	5.39 (t, 9.9, 10.7)
5	76.7	3.94 (m)
6	62.3	4.36 (d, 12.1) 4.19 (m)
Galloyl A		
1a	118.9	
2a, 6a	108.9	6.88 (2H, s)
3a, 5a	145.1	
4a	138.8	
7a	165.8	
Galloyl B 1b		
	119.2	
2b, 6b	108.9	6.82 (2H, s)
3b, 5b	144.8	
4b	138.6	
7b	166.2	
Galloyl C		
1c	118.9	
2c, 6c	109.0	6.87 (2H, s)
3c, 5c	145.0	
4c	138.8	
7c	165.6	
Galloyl D		
1d	119.7	
2d, 6d	108.8	7.00 (2H, s)
3d, 5d	145.0	
4d	138.5	
7d	166.6	

Figure 1. Key HMBC correlations of maplexin J.

CHAPTER 3

ELLAGITANNINS

Manuscript 4 appears as published:

Pomegranate's Neuroprotective Effects against Alzheimer's Disease are Mediated by Urolithins, its Ellagitannin-Gut Microbial Derived Metabolites. Yuan, Tao; Ma, Hang; Liu, Wang; Niesen, Daniel B.; Shah, Nishan; Crews, Rebecca; Vatter, Dhiraj A.; Seeram, Navindra P. (2016). <i>ACS Chemical Neuroscience</i> . 7, 26-33.....	116
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ABSTRACT

Previous reports have demonstrated the ability of pomegranate extract (PE) and pomegranate juice (PJ) to attenuate Alzheimer's disease (AD) pathogenesis in several transgenic animal models, as well as aiding in memory related tasks in humans [1-4]. Collectively, these findings suggest a role for pomegranate to augment memory function through task-related activities. The pomegranate fruit is a rich source of ellagitannins, most notably punicalagin. However, upon ingestion, ellagitannins are poorly absorbed in the gut and are quickly hydrolyzed releasing their hallmark 'hexahydroxydiphenyl' substituent, which quickly undergoes an internal lactonization to generate ellagic acid. Unfortunately, much like its parent compound, ellagic acid is not readily absorbed and does not reach physiologically relevant plasma concentrations. Ellagic acid is metabolized by the gut microflora into dibenzopyranone derivatives, known as urolithins. The urolithins, most notably urolithin A (UA; 3,8-dihydroxy-6H-dibenzo(b,d)pyran-6-one) achieves biologically significant concentrations (20-100 μ M) in plasma and select tissues [5-7]. As ellagitannins are the major class of compounds found in pomegranate, we therefore hypothesize that the urolithins could be the neurologically active compounds responsible for the link between pomegranate and AD.

Our first priority was to synthesize several of the most physiologically relevant urolithin derivatives including urolithin A (3,8-dihydroxybenzo[b,d]pyran-6-one), methyl-urolithin A (3-methoxy-8-hydroxybenzo[b,d]pyran-6-one), urolithin B (3-hydroxy-6H-dibenzo[b,d]pyran-6-one) and methyl-urolithin B (3-methoxy-6H-

dibenzo[b,d]pyran-6-one). Using these synthetic standards, LC-MS/MS methods have been developed in conjunction with cellular, tissue, and *Caenorhabditis elegans*, uptake studies.

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MANUSCRIPT 4

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Pomegranate's Neuroprotective Effects against Alzheimer's Disease are Mediated by Urolithins, its Ellagitannin-Gut Microbial Derived Metabolites. Yuan, Tao; Ma, Hang; Liu, Wang; Niesen, Daniel B.; Shah, Nishan; Crews, Rebecca; Vatter, Dhiraj A.; Seeram, Navindra P. (2016). *ACS Chemical Neuroscience*. 7, 26-33.

Abstract

Pomegranate shows neuroprotective effects against Alzheimer's disease (AD) in several reported animal studies. However, whether its constituent ellagitannins and/or their physiologically relevant gut microbiota-derived metabolites, namely, urolithins (6Hdibenzo[b,d]pyran-6-one derivatives), are the responsible bioactive constituents is unknown. Therefore, from a pomegranate extract (PE), previously reported by our group to have anti-AD effects *in vivo*, 21 constituents, which were primarily ellagitannins, were isolated and identified (by HPLC, NMR, and HRESIMS). *In silico* computational studies, used to predict blood-brain barrier permeability, revealed that none of the PE constituents, but the urolithins, fulfilled criteria required for penetration. Urolithins prevented β -amyloid fibrillation *in vitro* and methyl-urolithin B (8-methoxy-6H-dibenzo[b,d]pyran-6-one), but not PE or its predominant ellagitannins, had a protective effect in *Caenorhabditis elegans* post induction of amyloid β_{1-42} induced neurotoxicity and paralysis. Therefore, urolithins are the possible brain absorbable compounds which contribute to pomegranate's anti-AD effects warranting further *in vivo* studies

1. Introduction

Alzheimer's disease (AD) is a degenerative brain disease that is projected to affect over 115 million people worldwide by 2050. AD is a leading cause of disability and morbidity among patients and is among the most costly chronic diseases known to society. Apart from its public health burden, the economic cost of AD exceeded 200 billion dollars for 2014 alone which is estimated to increase 5-fold by 2050 [1]. If the incidence of AD continues on its current trajectory, it will cripple the health care systems of several countries including the United States where it is the sixth leading cause of death [2]. Unfortunately, despite several decades of research, many approved drugs for AD have little effect on slowing disease progression. In fact, it is estimated that the brain changes for AD may begin more than 20 years before symptoms of the disease appear and it is often too late to reverse AD pathology by the time of diagnosis. Therefore, it is imperative that other approaches, such as the utilization of natural products as dietary intervention strategies, be explored as preventive and/or disease-modifying measures to slow or stop AD progression.

Among natural compounds, plant polyphenols have emerged as an important nonpharmacologic approach for AD prevention and treatment [3,4]. However, the majority of polyphenols, including the subclass known as ellagitannins, are poorly absorbed in the small intestine and do not achieve physiologically relevant concentrations in circulation [5,6]. Instead, they reach the colon where they are extensively metabolized by gut microbiota to colonic-derived metabolites, which are implicated with a vast array of biological effects [7,8]. Given considerable inter-

individual variability in microflora, and different phenotypes being observed with “metabolite-producers and non-producers” after consumption of many polyphenol subclasses, including ellagitannins [9]. Further investigations into understanding the biological effects of these colonic metabolites are necessary.

The pomegranate (*Punica granatum* L.) fruit is a rich source of ellagitannins, primarily punicalagin (PA) and its hydrolysis product, ellagic acid (EA) [10]. Pomegranate juice and extracts have been reported to show neuroprotective effects against AD pathogenesis in several transgenic animal models but the bioactive compound/s responsible have not been characterized [11–14]. Furthermore, the identity of the brain absorbable compounds, whether they are the natural ellagitannin constituents present in pomegranate, and/or their *in vivo* colonic-derived metabolites, is not known.

The bioavailability and metabolism of ellagitannins in human subjects, after the consumption of pomegranate juice and pomegranate extracts, are well established [5–6]. The major pomegranate ellagitannins, PA and others, are not found intact in circulation, but rather are hydrolyzed to release EA and then subsequently biotransformed by gut microbiota to yield urolithins (6H-dibenzo[b,d]pyran-6-one derivatives) (see Figure 1A). These urolithins and their phase-2 enzyme conjugates [methylated (i.e., conversion of hydroxyl to methoxyl or methyl ether), sulfated, and glucuronidated forms] [15] achieve physiologically relevant concentrations through enterohepatic recirculation and persist *in vivo* following the regular consumption of pomegranate foods [5–8]. Therefore, the poor bioavailability and extensive metabolism

of pomegranate ellagitannins to urolithins suggest that these latter metabolites may be relevant bioactive compounds *in vivo* [5]. Moreover, urolithins have been reported to show anti-inflammatory [5], and anti-glycative and neuroprotective effects *in vitro* [16]. However, it is also possible that there are unidentified compounds, yet to be isolated from pomegranate, which are responsible for its neuroprotective effects.

Our group has recently reported on the biological effects of an ellagitannin-enriched pomegranate extract (PE) in an aged AD transgenic animal model [17]. Therefore, from this PE, herein we sought to (1) isolate and identify its chemical constituents; (2) conduct *in silico* computational studies to evaluate whether the PE constituents and several urolithin analogues [urolithin A (UA) and urolithin B (UB) and their methyl derivatives, mUA, and mUB, respectively], can cross the blood-brain barrier (BBB); (3) evaluate the *in vitro* effects of PE, its constituents [PA, EA, and gallic acid (GA)], and the urolithins, on A β ₁₋₄₂ fibrillation and; (4) evaluate the *in vivo* ability of PE and the aforementioned pure compounds (constituents and urolithins) to abrogate A β ₁₋₄₂ induced neurotoxicity and paralysis in *Caenorhabditis elegans*. This is the first study to investigate a PE, its constituents, and the urolithins, for *in silico* BBB penetrability and *in vitro* and *in vivo* anti-AD potential.

Given that the PE was previously reported to show anti-AD effects in an animal model [17], we first sought to isolate and identify all of its constituents. Twenty-one compounds (see Figure 1B), predominantly ellagitannins, were identified from the PE (by HPLC, NMR, and mass spectral data; described in the Supporting Information).

The isolates included PA, EA, and gallic acid (GA) and other compounds common to pomegranate [10, 18]. In addition, a new ellagitannin was isolated and assigned the common name of pomellatannin (1). This compound is a dehydroellagitannin acetone condensate, and its structure is in accordance to similar ellagitannin-acetone derivatives previously reported [19]. The remaining isolates 2–17 were identified based on ^1H NMR and/or ^{13}C NMR data and by comparison of these data to published literature reports as follows: punigluconin (2) [20], 6-O-galloyl-D-glucose (3) [21], gemin D (4) [22], hippomanin A (5) [22], praecoxin B (6) [23], pedunculagin (7) [24], 1,6-di-O-galloyl- β -D-glucose (8) [21], gallic acid-3-O- β -D-(6'-O-galloyl)-glucopyranoside (9) [21], isocorilagin (10) [25], casuariin (11) [26], ellagic acid-4-O- β -D-glucopyranose (12) [27], 3,3'-di-O-methyl-ellagic acid-4-O- β -D-glucopyranose (13) [28], 4-O- α -L-rhamnopyranosyl-ellagic acid (14) [29], 4-O- α -L-arabinofuranosyl-ellagic acid (15) [30], gallocatechin (16) [31], and brevifolincarboxylic acid (17) [32].

Having obtained the structures of the natural constituents present in the PE, which were primarily ellagitannins, we next sought to investigate whether the isolates and their colonic derived microbial metabolites, namely urolithins, could potentially cross the BBB. Therefore, using *in silico* computational methods as previously reported [33], the 21 constituents identified in PE, as well as UA, UB, mUA, and mUB, were evaluated for BBB penetrability. Interestingly, none of the isolates, but all of the urolithins, fulfilled criteria required for BBB penetration (Table 1).

The methyl derivatives of UA and UB (i.e., mUA and mUB) may have *in vivo* relevance since these compounds could be formed from the metabolism of UA and UB by phenol-O-methyltransferase, a mammalian enzyme which is highly localized in the liver, and can transfer the methyl group of S-adenosylmethionine to phenols [34]. Indeed, mUA has previously been detected in tissues of mice after oral delivery of UA [35]. However, apart from these methyl derivatives, we also evaluated other potential mammalian enzyme-biotransformation products, namely, sulfated and glucuronidated derivatives of UA and UB for BBB penetrability. Interestingly, while none of these latter metabolites fulfilled criteria required for BBB penetration, the dimethyl derivative of UA (3, 8-dimethoxy-6H-dibenzo[b,d]pyran-6-one) did fulfill the criteria required for BBB penetration (data not shown). Therefore, it is possible that the increased lipophilicity caused by methylation of the hydroxyl group/s on UA and UB (to yield the methyl ether/methoxyl derivatives) increases BBB penetrability unlike increased hydrophilicity which is imparted by sulfation or glucuronidation. Methylated conjugates of other polyphenols, including monomeric flavanols (catechins), have been reported to remain intact and persist *in vivo* since they are not susceptible to enzymatic deconjugation unlike sulfated and glucuronidated metabolites which can be deconjugated *in vivo* by sulfatases and β -glucuronidases, respectively [36].

Apart from the dietary relevance of the urolithins and their conjugates due to their formation from ellagitannins by colonic microbiota, their subsequent biotransformation by mammalian enzymes, and their persistence *in vivo* through enterohepatic circulation [5–8], they could also be explored for pharmaceutical potential given that structural

analogues, and accompanying SAR (structure–activity related) studies, can yield compounds with enhanced activity and BBB penetrability.

It is necessary, though, that the *in silico* data reported herein be substantiated by future animal brain tissue disposition studies. Interestingly, a recent report (using mass spectroscopic methods) has detected UB in brain tissues of rats after intravenous delivery [37]. However, animal studies to evaluate brain deposition after repeated oral exposure of PE, as well as the individual urolithin analogs, is warranted. These studies are necessary since it has been reported that the brain bioavailability of certain polyphenols, such as monomeric flavanols (catechins), can increase after repeated oral dosing as has been observed with a polyphenol-rich grape seed extract [38]. Next, biophysical methods were used to evaluate the effects of PE, its constituents (PA, EA, and GA), and the urolithins on A β fibrillation. This is because elevated levels of A β fibrillation and oligomerization in brain are associated with neurotoxicity in AD and are characteristic hallmarks which play significant roles in both early and late stages of AD [39, 40]. Therefore, agents which target the formation of A β fibrils and oligomers could serve as therapeutic approaches for AD prevention and/or treatment. A β _{1–42} fibrillation was confirmed by the ThT assay which showed a significant increase in fluorescence which was then correlated to binding levels of ThT to A β fibril content, β -sheet formation [40] and peptide oligomerization [39]. The PE treated samples reduced A β fibrillation by 35.9% and 76.4%, at 10 and 100 μ g/mL, respectively. The purified PE constituents and urolithins reduced A β fibrillation at levels ranging 6.5–65.4% (at 10 μ M) and 20.2–76.3% (at 100 μ M) (Figure 2). These inhibitory levels

were similar to those of resveratrol (37.6% at 10 μ M and 74.4% at 100 μ M), the well-known grape/red wine polyphenol, which has also been previously reported to reduce A β fibrillation *in vitro* [41]. Therefore, the preventive abilities of the urolithins on the assembly of neurotoxic A β fibril structures may contribute to the overall neuroprotective effect reported for pomegranate but further studies would be required to confirm this.

Lastly, the PE was evaluated, its purified constituents, and the urolithins (all at 10 μ g/mL), using an *in vivo* *C. elegans* model of AD. The mobility curves for the CL4176 *C. elegans* strain after the A β _{1–42} induction of muscular paralysis at 25°C are shown in figure 3 and the mean, maximum, and median survival of the worms post heat shock are shown in table 2. Compared to the control worms, treatment with PE (Figure 3B) did not have any significant effect on the mean, maximum, or median survival/mobility in *C. elegans* post induction of A β _{1–42} induced neurotoxicity and paralysis. Treatment with EA (Figure 3D), UB (Figure 3F), mUA (Figure 3G), and GA (Figure 3I) did not have any effect on the mean or medium survival/mobility in *C. elegans* but significantly ($p < 0.0001$) increased the maximum survival/mobility by 10.6, 8.5, 12.6, and 16.4%, respectively (Table 2). Among all of the samples, only treatment with mUB (Figure 3H) significantly ($p < 0.0001$) increased mean, maximum, and median survival/mobility in *C. elegans* post induction of A β _{1–42} induced neurotoxicity and paralysis by 5.6, 13.0, and 10.3% respectively (Table 2). Treatment with PA (Figure 3C) and UA (Figure 3E) did not have any significant effect on the maximum or median survival/ mobility, but significantly ($p < 0.0001$) decreased the mean survival/mobility

in *C. elegans* post induction of amyloid β _{1–42} induced neurotoxicity and paralysis by 5.6% (Table 2).

The uptake of urolithins, specifically UA and UB, has previously been reported in different cell lines [42, 43], but to date, similar studies have not been conducted with *C. elegans*. Therefore, using a protocol reported for *C. elegans* uptake studies with the polyphenol, quercetin [44], wild type N2 nematodes were exposed to UA and subsequent liquid chromatography mass spectroscopy (LC-MS/MS) analyses revealed its uptake into the tissues of the worms. However, further quantitative and metabolism studies of urolithins in *C. elegans* are warranted.

In summary, we isolated and identified the naturally occurring constituents present in a PE previously reported to have anti-AD effects in an animal model [17]. None of these compounds, but urolithins (gut microbial metabolites derived from ellagitannins), fulfilled *in silico* criteria required for BBB penetration. Moreover, the urolithins prevented β -amyloid fibrillation *in vitro* and methyl-urolithin B, but not PE or its constituents, protected *C. elegans* post induction of A β _{1–42} induced neurotoxicity and paralysis. Therefore, further studies to evaluate the neuroprotective effects of urolithins and their structural analogs in animal models of AD are warranted.

2. Experimental

2.1 Pomegranate Extract (PE).

The pomegranate extract (PE) used for the isolation studies is of the same lot number as the PE recently reported by our group to show anti-AD effects in a transgenic animal model [17]. The PE is a whole pomegranate fruit extract (Pomella) provided by Verdure Sciences (Noblesville, IN) standardized to PA (ca. 30%) and EA (2.3%).

2.2 Isolation and Identification of Compounds from the PE.

Details of the isolation and identification of the compounds are provided in the Supporting Information

2.3 Urolithins.

Urolithins (6H-dibenzo[b,d]pyran-6-one derivatives) including urolithin A (3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one; UA), methyl-urolithin A (3-hydroxy-8-methoxy-6H-dibenzo[b,d]pyran-6-one; mUA), urolithin B (8-hydroxy-6H-dibenzo[b,d]pyran-6-one; UB), and methyl-urolithin B (8-methoxy-6H-dibenzo[b,d]pyran-6-one; mUB) were synthesized in our laboratory according to previously reported methods [45]. Their structures were verified by NMR and mass spectral analyses and they are all >98% purity.

2.4 In Silico Computational Approach.

Using previously reported methods [33], BBB penetrability data including brain transfer descriptors (Table 1) were obtained using prediction software developed by ACD Laboratories (Toronto, Ontario, Canada).

2.5 A β _{1–42} Thioflavin T (ThT) Binding Assay.

Thioflavin T binding assay was used to measure human A β _{1–42} fibril formation as reported previously [39]. Briefly, A β _{1–42} peptide was dissolved in ammonium hydroxide, lyophilized, and redissolved in PBS buffer to obtain a final concentration of 50 μ M. Treatments included 10 or 100 μ g/mL of PE, and 10 or 100 μ M of PE constituents (PA, GA, and EA), urolithins (UA, UB, mUA, and mUB), and the positive control, resveratrol. After 10 days incubation at 37°C, 20 μ L of each sample was added to 100 μ L of ThT solution (10 μ M) and fluorescence was measured using a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 450 and 483nm, respectively. To evaluate the inhibitory effects on ThT binding, the activity of each treatment was expressed as a percent inhibition value (% inhibition) relative to the negative control. Percent inhibition was calculated by [(FU of negative control – FU of treated solution)/ FU of negative control] $\times$ 100% based on arbitrary fluorescence (FU). Statistical significance was analyzed by one-way factorial ANOVA with Tukey–Kramer post hoc comparisons. A significance value of $p < 0.05$ were set to evaluate the group difference, $n = 3$.

2.6 *C. elegans* Strains, Maintenance, and Assays.

Transgenic *C. elegans* strain CL4176, developed to express human amyloid β_{1-42} in the muscle tissue in response to heat shock [46], were obtained from the *Caenorhabditis* Genetics Center (CGC) (University of Minnesota, Minneapolis, MN). Worms were grown and maintained at 16°C on 60mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl, 0.25% peptone, 1mM CaCl₂, 1mM MgSO₄, 5mg/L cholesterol, 2.5mM KPO₄ at 16–20°C). Media was poured aseptically into culture plates (10mL for 60mm) using a peristaltic pump and allowed to solidify for 36h. NGM culture plates were then inoculated with 50 μ L of *Escherichia coli* OP50 (CGC, University of Minnesota, Minneapolis, MN) overnight cultures and incubated for 8h at 37°C. Strains of *C. elegans* were maintained by picking 2–3 young adult worms onto freshly inoculated NGM plates every 4–7 days.

2.7 Age Synchronization of *C. elegans*.

Prior to the beginning of the experiment, *C. elegans* were age synchronized as previously described [47, 48]. Briefly, 10 worms at L4 stage (F0) were transferred to single NGM plates and incubated at 16°C until they progressed to adulthood and laid eggs. Adults were immediately removed from the plates and the eggs were allowed to hatch (F1) and grow to L4 at 16°C. L4 worms of the F1 generation were again transferred to fresh NGM plates and allowed to mature into gravid adults and lay eggs at 16°C. Adults were quickly removed from the plates and returned to a 16 °C incubator to facilitate egg hatching. L1 worms (F2 generation) were collected from the plate by washing with S-basal buffer (0.59% NaCl, 5% 1M KPO₄, 5mg/mL cholesterol in

ethanol) into a sterile 15mL centrifuge tube. Worms from a minimum of five plates were pooled into a single centrifuge tube and centrifuged at 8000rpm for 10min at 10°C. The supernatant was carefully aspirated and the worms were washed again in S-basal buffer, centrifuged, and aspirated to leave approximately 1mL of S-basal buffer in the centrifuge tube. The tube was gently agitated to disperse the worms and 20µL was pipetted onto a slide and the number of worms was counted under a stereo microscope. The concentration of the worms was adjusted to 10–15 worms by diluting with S-complete liquid media (97.7% S-basal, 1% potassium citrate, 1% trace metals, 0.3% CaCl₂, 0.3% MgSO₄). A 100mg/mL suspension of *E. coli* OP50 was prepared by centrifuging 100mL of an overnight *E. coli* OP50 culture in LB broth at 3500rpm. Spent LB broth was aspirated and pellet was washed several times by resuspension and centrifugation in sterile distilled water. The weight of the resultant pellet was determined and adjusted to 100mg/mL using S-complete medium.

2.8 AD Assay and Treatments in Transgenic C. elegans.

Prior to the beginning of the experiment, *C. elegans* were age synchronized at 16°C and L1 worms from the F2 generation were transferred to control or treatment plates and allowed to mature gravid adult stage to lay eggs. For *C. elegans* treatment, stock solutions (1mg/mL) of the samples were prepared as follows: PE and PA were dissolved in a 1:1 (v/v) mixture of S-basal buffer and methanol and diluted in S-basal buffer to a final concentration of 1mg/mL. EA, UA, UB, mUA, and mUB were dissolved in DMSO and diluted in S-basal buffer to a final concentration of 1 mg/mL. GA was dissolved in methanol and diluted in S-basal buffer to a final concentration of

1mg/mL. For preparing the treatment plates, stock solutions of PE, PA, EA, GA, UA, UB, mUA, and mUB were added directly to the NGM media to obtain a final concentration of 10µg/mL. A test concentration of 10 µg/mL (i.e., 10ppm) was selected for these assays since the test samples included an extract (i.e., PE) along with pure compounds. However, for the pure compounds, the 10µg/mL concentration is equivalent to the following µM concentrations: PA = 9.2µM; EA = 33.1µM, GA =58.8µM, UA = 43.8µM, mUA = 41.3µM, UB = 47.2µM, and mUB = 44.2µM. Control and treatment NGM plates were then inoculated with 25µL of *Escherichia coli* (*E. coli*) OP50 suspension (100mg/mL) and incubated for 24h at 23°C. The OP50 used for inoculation of treatment plates also contained the different treatments at a final concentration of 10µg/mL. To standardize the food supply, the plates were then incubated under UV light in a Stratagene UV Stratalinker 2400 (La Jolla, CA) at maximum dose for 5 min to arrest growth of the *E. coli* OP50. Upon development of the eggs to the L3 stage, the incubation temperature of the plates was increased from 16 to 25°C, in order to induce the expression of amyloid β_{1-42} [46]. Mobility scoring was conducted beginning 20h after temperature upshift and continued in 2 h increments until all of the worms were paralyzed. Three replicates per experiment were performed with a minimum of 200 worms. Failure to respond to touch (prodding with a worm pick) and absence of pharyngeal pumping were used to score paralyzed/dead worms. Survival curves were plotted to calculate the mean, median, and maximum survival of post heat shock treatment.

2.9 Statistical Analyses.

Results are expressed as mean $\pm$ standard deviation. For the AD assay, the Kaplan–Meier method was used to compare the lifespan survival curves and the survival differences were tested for significance ($p < 0.05$) using the Log rank test (Mantel Cox). Both tests used GraphPad Prism software 6.0 (GraphPad Software, Inc., San Diego, CA).

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Table 1. BBB Penetrability of Compounds Present in the PE and Urolithins (UA, UB, mUA, and mUB) Using Computational Methods and Software Developed by ACD/Labs (Toronto, Ontario, Canada)

compd	log P	pK _a	fraction unbound in plasma	penetration rate (log PS)	penetration extent (log BB)	sufficient BBB penetration
1	-0.88	6.10	0.64	-8.10	-2.00	no
2	0.76	1.90	0.27	-8.20	-2.00	no
3	-1.26	7.80	0.76	-5.00	-2.00	no
4	0.56	6.10	0.52	-6.90	-2.00	no
5	0.67	6.10	0.41	-6.80	-2.00	no
6	1.82	6.10	0.36	-7.10	-2.00	no
7	1.64	5.80	0.34	-7.40	-2.00	no
8	-0.33	7.50	0.71	-5.50	-2.00	no
9	-0.07	4.30	0.22	-6.60	-2.00	no
10	0.80	6.10	0.53	-6.70	-2.00	no
11	0.60	5.80	0.46	-8.10	-2.00	no
12	-0.08	7.00	0.49	-4.70	-0.27	no
13	-0.58	6.60	0.38	-5.80	-2.00	no
14	-0.10	6.60	0.30	-5.20	-2.00	no
15	0.80	6.60	0.57	-5.10	-2.00	no
16	0.27	8.70	0.30	-3.60	-0.42	no
17	0.14	3.50	0.40	-4.90	-0.41	no
punicagalin	3.20	5.70	0.07	-8.30	-2.00	no
punicalin	0.69	5.20	0.26	-8.00	-2.00	no
ellagic acid	1.91	6.30	0.35	-3.70	-0.16	no
gallic acid	0.65	4.30	0.17	-4.20	-0.71	no
UA	2.87	9.00	0.09	-1.40	0.01	yes
UB	2.98	9.10	0.08	-1.20	0.03	yes
mUA	3.54	9.10	0.09	-1.60	0.38	yes
mUB	3.81	None	0.02	-1.10	-0.03	yes

Table 2. Survival (Mean, Median, and Maximum) of (CL4176) *C. elegans* Worms Treated with PE or Pure Compounds (10µg/ mL) 20h Post Aβ₁₋₄₂ Induction of Muscular Paralysis at 25°C^a

survival (h)	ctrl	concn (10 µg/mL)							
		PE	PA	EA	GA	UA	UB	mUA	mUB
mean	29.45	28.20	27.80*	28.30	29.94	27.80*	30.00	29.90	31.11*
maximum	29.47	31.70	31.10	32.60*	34.35*	29.50	32.00*	33.02*	33.33*
median	29.00	28.00	28.00	28.00	30.00	28.00	30.00	30.00	32.00*

^aNote: p < 0.05 log rank test (Mantel Cox).

Figure 1. (A) Chemical structures of punicalagin (PA) and ellagic acid (EA) and their gut microbial metabolites, urolithins. (B) Chemical structures of compounds identified in the pomegranate extract (PE).

Figure 2. Inhibition on A β_{1-42} fibrillation measured by the ThT binding assay. Treatments include 10 and 100 μ M of PE constituents (PA, EA, and GA), urolithins (UA, UB, mUA, and mUB), and the positive control, resveratrol (Resv). The inhibition level on ThT binding of each treated solution was expressed as a percent inhibition value (% inhibition) relative to the negative control. % inhibition was calculated based on arbitrary fluorescence (FU) using the following equation: % inhibition = [(FU of negative control – FU of treated solution)/FU of negative control] $\times$ 100%. Data was obtained from triplicate experiments (n = 3).

Figure 3. Mobility curves of transgenic (CL4176) *C. elegans* 20 h post $A\beta_{1-42}$ induction of muscular paralysis at 25 °C. Kaplan–Meier mobility plots of *C. elegans* worms fed on (A) control [NGM]; (B) PE [NGM + 10 μ g/mL PE; pomella extract]; (C) PA [NGM + 10 μ g/mL punicalagin]; (D) EA [NGM + 10 μ g/mL ellagic acid]; (E) UA [NGM + 10 μ g/mL urolithin A]; (F) UB [NGM + 10 μ g/mL urolithin B]; (G) mUA [NGM + 10 μ g/mL methyl-urolithin A]; (H) mUB [NGM + 10 μ g/mL methyl-urolithin B]; and (I) GA [NGM + 10 μ g/mL gallic acid].

Synthesis of Urolithins

The synthesis of urolithin A was completed as the literature dictated [45] (Scheme 1). Briefly, 2-bromo-5-methoxybenzoic acid (1, 2.00g), was brought to reflux in basic (NaOH) water (15ml, pH 13.). Resorcinol (2, 5.00g) was added to the reaction and reflux continued for 30 minutes. Aqueous CuSO₄ (5% w/v, 1.80ml) was then added and the solution was refluxed for an additional 10 minutes. During this addition, 8-O-methylurolithin A (3) began to lightly precipitate out as a light yellow/white solid. The resulting slurry was extracted with ethyl acetate (3 x 25mL). The combined organic layers were then washed with a saturated brine solution, and then dried over sodium sulfate. The solution was filtered and solvent was removed *in vacuo*. The resultant product was washed with cold methanol to remove any leftover starting materials and afforded pure 8-O-methylurolithin A (Figure 1). 8-O-methylurolithin A (3, 0.040g) was dissolved in 0.5ml anhydrous dichloromethane and 1M BBr₃ in dichloromethane (700uL) was added over several minutes. After 18 hours, the reaction was quenched through dropwise addition of distilled water. The resultant slurry was extracted with ethyl acetate (3 x 25mL). The combined organic layers were then washed with a saturated brine solution, and then dried over sodium sulfate. The solution was filtered and solvent was removed *in vacuo* to afford urolithin A (Figure 2).

Urolithin B (5) was synthesized similarly to 8-O-methylurolithin A, with slight modifications in the starting materials (Scheme 2). Briefly, urolithin B requires the starting material 2-bromobenzoic acid (6), instead of 2-bromo-5-methoxybenzoic acid (1), however the reaction was carried out in the same manner. Urolithin B was isolated

using silica gel column chromatography using an isocratic solvent system of 25:75 ethyl acetate:hexanes. Once urolithin B was synthesized and isolated (Figure 3), successful methylation of the phenol was achieved using excess methyl iodide and lithium bicarbonate at room temperature in dimethylformamide to produce methyl-urolithin B (3-methoxy-6H-dibenzo[b,d]pyran-6-one) (7) (Scheme 2). The resultant product was washed with cold methanol to remove any leftover starting materials and afforded pure methyl-urolithin B (Figure 4).

UFLC-MS/MS optimization

Metabolite identification using UFLC-MS/MS was measured using a Shimadzu UFLC system (3 LC-20AD pumps, Degasser DGU-20A_{5R}, autosampler SIL-20AC HT, column oven CTO-20AC, Analyst v1.6.2) coupled to an ABSCIEX QTrap 4500 mass spectrometer using an electrospray source interface. Identification and separation was achieved on a reverse phase Phenomenex C18 column (250 × 4.6mm, 5µm) operating at 40°C. The mobile phases were water:formic acid (99.9:0.1 v/v; phase A) and methanol:formic acid (99.9:0.1 v/v; phase B). Gradient program was as follows: 0–20min, 50-100% B; 20–25min, 100% B; 25–27min, 100–50% B, 27– 32min, 50% B. Using the synthesized standards, optimization of the MS/MS fragmentation of the urolithins was possible. Urolithin A was optimized over each of the specific 5 transitions: [M+H] 229-195, 229-157, 229-139, 229-128, 229-114.9. Each of the transitions is represented by a specific color: 195 blue, 157 red, 139 green, 128 grey, 114.9 pale blue (Figure 5).

Uptake of Urolithin A by *C. elegans*

A confluent plate of wild type N2 nematodes grown in NGM/OP50 (1.7% Agar, 0.3% NaCl, 0.25% Peptone, 1mM CaCl₂, 1mM MgSO₄, 5mg/L cholesterol, 2.5mM KPO₄) (100μL of overnight OP50 E. coli culture) were washed from the plate with two washes of 5mL of S-Basal buffer (0.59% NaCl, 5% KPO₄, 5mg/ml cholesterol) and transferred to a 15mL centrifuge tube. Then 100μL of a 50μM solution of Urolithin A (0.05% DMSO and Water) was added to the nematodes/buffer solution. The nematodes were incubated at 20°C for 4 hours. After incubation the nematodes were centrifuged at 1200 rpm for five minutes. The supernatant was decanted and the nematodes were washed three times with 10mL of PBS +1% BSA, 10mL of PBS + 0.01% Tween20, and 10mL PBS. After the final wash the supernatant was decanted and the dry pellet was stored at -80°C.

1mL of 50:49.9:0.1 (MeOH:H₂O:HCl, v/v) was added to the samples and stored in -80°C until completely frozen into a solid. Samples were removed from the freezer and sonicated for 15 minutes in an ice water bath, or until the sample had melted. Three rounds of freezing/sonication took place to ensure the rupture of the *C. elegans*. Samples were centrifuged (Eppendorf Centrifuge 5804) at 5,000rpm for 5 minutes to pellet worm body material and protein pieces. The supernatant was collected and dried down *in vacuo*. 1mL of deionized water was added and the samples were sonicated for 10 minutes. The samples were eluted through a C18 SPE (Alltech, 50mg, 1mL) column. Prior to the addition of the sample, the C18 SPE column was rinsed with 5mL of HPLC grade methanol, followed by 2mL of deionized water.

1mL of additional water was added to the column to elute a total of 2mL. 2mL of tetrahydrofuran (THF) was eluted through the column. The organic layer was collected separately and dried *in vacuo*. The dried organic layer was reconstituted in 200mL of THF and 10uL was injected into the UFLC-MS/MS. Each of the mass transitions is represented by a specific color: 195 blue, 157 red, 139 green, 128 grey, 114.9 pale blue. Our untreated group can be found in Figure 6, while our *C. elegans* treated with urolithin A can be found in figure 7.

Polyphenol Microbial Metabolites

In addition to ellagitannin metabolism into benzopyranone derivatives, there are other relationships between dietary polyphenols and bacterially metabolized endproducts [1-3]. It is known that the metabolites of lignans, a common dietary polyphenol found in flaxseed, are two smaller polyphenols enterodiol and enterolactone [2]. Additionally it was identified in the 1940s that isoflavones, found in high concentrations in soy, require bacteria to be metabolized into equol [4]. Therefore, in addition to the development of analytical methods for the study of ellagitannins and urolithins, UFLC-MS/MS fragmentation to study the uptake of other polyphenol microbial metabolites of interest, as well as other notable dietary polyphenols, has been established (Table 1).

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Scheme 1. Synthesis of methyl-urolithin A (3-methoxy-8-hydroxybenzo[b,d]pyran-6-one) (3) and urolithin A (3,8-dihydroxybenzo[b,d]pyran-6-one) (4) from 2-bromo-5-methoxybenzoic acid (1) and resorcinol (2).

Scheme 2. Synthesis of methyl-urolithin B (8-methoxybenzo[b,d]pyran-6-one) (5) and urolithin B (8-hydroxybenzo[b,d]pyran-6-one) (7) from 2-bromobenzoic acid (6) and resorcinol (2).

Table 1: MS/MS fragmentation of other polyphenol microbial metabolites of interest, as well as other notable dietary polyphenols. MS³ designated by (*), MS⁴ designated by (+).

Polyphenol Name	Mass Transition
Secoisolariciresinol	[M+H] 363.42, 345, 295, 327*, 263*, 163*, 137+, 133*
Enterodiol	[M+H] 303.37, 285, 267*, 147 ⁺ , 133 ⁺ , 107 ⁺
Enterolactone	[M+H] 299.34, 281*, 263 ⁺ , 133 ⁺ , 107*
Daidzein	[M+H] 255.23, 237, 227, 199*, 181*, 137
Equol	[M+H] 243.3, 149, 133, 123, 107
Quercetin	[M+H] 303.24, 257, 229*, 165, 153, 137
Curcumin	[M+H] 369.39, 284, 253*, 245, 177, 145*
Desmethoxycurcumin	[M+H] 339.36, 255, 223*, 177, 145*
Punicalagin	[M+H] 1085.7, 106.78, 783, 765*, 621*, 603*, 557 ⁺ , 303 ⁺
Punicalin	[M+H] 783.52. 765, 603*, 557 ⁺ , 303 ⁺
Ellagic Acid	[M+H] 303.2, 285, 275, 257*, 247*, 229 ⁺ , 201 ⁺

.p

Figure 2. ^1H NMR of synthesized urolithin A (3,8-dihydroxybenzo[b,d]pyran-6-one). Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

Figure 3. ^1H NMR of synthesized methyl-urolithin b (8-methoxybenzo[b,d]pyran-6-one). Data was measured in CH_3OD at 500 MHz (^1H) and 125 MHz (^{13}C).

Figure 4. ¹H NMR of synthesized urolithin b (8-hydroxybenzo[b,d]pyran-6-one). Data was measured in CH₃OD at 500 MHz (¹H) and 125 MHz (¹³C).

Figure 5. UFLC-MS/MS transitions of the fragmentation of UA standard. Each transition is represented by a specific color: [M+H]⁺ 229-195 blue, 229-157 red, 229-139 green, 229-128 grey, 229-114.9 pale blue.

Figure 6. UFLC-MS/MS transitions of the fragmentation of untreated worms. Each MS/MS transition of UA is represented by a specific color: [M+H]⁺ 229-195 blue, 229-157 red, 229-139 green, 229-128 grey, 229-114.9 pale blue.

Figure 7. UFLC-MS/MS transitions of the fragmentation of worms treated with UA. Each MS/MS transition of UA is represented by a specific color: [M+H]⁺ 229-195 blue, 229-157 red, 229-139 green, 229-128 grey, 229-114.9 pale blue.

CONCLUSIONARY REMARKS

Well known medicinal chemist and text book author, Dr. Richard Silverman, describes medicinal chemistry in The Organic Chemistry of Drug Design and Delivery:

“Medicinal Chemistry may involve isolation of compounds from nature or the synthesis of new molecules, investigations of the relationships between the structure of natural and/or synthetic compounds and their biological activities, elucidations of their interactions with receptors of various kinds, the determination of their absorption, transport and distributions properties, and studies of metabolic transformations.”

Dr. Silverman’s lengthy description of medicinal chemistry highlights how diverse the studies of drug discovery, and drug development, can be during the preclinical stages. The work provided in this dissertation highlights several key elements of Dr. Silverman’s definition, including the isolation of compounds from nature, the synthesis of new molecules, elucidations of the interactions between receptors and their ligands, and studies of metabolic transformations.

The phytochemical investigation of *Carex vulpinoidea* (Fox Sedge) in pursuit of novel stilbenes concluded with the identification and structural elucidation of two new monomeric stilbenes, namely, vulpinoideols A and B. A synthetic route for naturally isolated gallotannins, namely, maplexins F and J, previously isolated from red maple (*Acer rubrum*) tree bark, was developed. Additionally, the ligand-enzyme interaction between the gallotannins and their target enzyme, α -glucosidase, was evaluated using biophysical tools. Finally, a synthetic route was also developed for the ellagitannin gut-

microbial metabolites, urolithins (dibenzopyranone derivatives), along with analytical UFLC-MS/MS methods to study the cellular and tissue uptake of the urolithins, as well as other dietary polyphenolic metabolites of interest.

While today's pharmaceuticals trend towards increasing the generation and administration of larger macromolecules, straying from the traditional small molecules, I believe that there will continue to be a demand for novel small molecule driven research. Traditionally, the research has been motivated by a single compound driving a single biological response, however I believe the future monumental discoveries of small molecules and natural products will involve the complex interactions and diversity of compounds found in botanical extracts. Even now while conclusive data on the effectiveness, pharmacological, and toxicological data is difficult to identify, dietary polyphenols continue to attract both media and research attention. Additionally, we are only just beginning to scratch the surface of knowledge into the gut microbiota. In the future lies extensive analytical and instrumental advances, which will allow us to tease apart the complex biochemical relationships, and their metabolisms.

R.B. Silverman and M.W. Holladay. The organic chemistry of drug design and drug delivery. Elsevier Inc, San Diego California, USA. 2014.