Estrogenicity and metabolism of prenylated flavonoids and isoflavonoids

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Abstract

Binding of (prenylated) flavonoids and isoflavonoids to the human estrogen receptors (hERs) might result in beneficial health effects *in vivo*. To understand structure-activity relationships of prenylated (iso)flavonoids towards the hERs, prenylated (iso)flavonoids were purified from extracts of licorice roots and elicited soybean seedlings. It was observed that prenylation can modulate estrogenicity. Unprenylated, chain and δ-position pyran prenylated (iso)flavonoids show an agonistic mode of action, whereas α/β-position pyran, α/β-position furan and double chain prenylated (iso)flavonoids show an antagonistic mode of action towards hERα in the yeast bioassay. The mode of estrogenic action of prenylated (iso)flavonoids could be related to structural features of the hER. In particular, the increase in length of α/β-position pyran prenylated compounds was related to indirect antagonism. It was also shown that heat and acid affected the stability of 6a-hydroxy-pterocarpans, converting them into their respective 6a,11a-pterocarpenes and consequently modulate their estrogenicity. Six prenylated isoflavonoids acted as SERMs and eight prenylated isoflavonoids showed ER subtype-selective behavior. The kind of prenylation (chain, furan or pyran) was most important for determining SERM activity, whereas additionally the backbone structure, *i.e.* the presence of an additional D-ring, was of importance for determining ER subtype-selectivity. To determine structure-metabolism relationships, *in vitro* conversion of purified prenylated (iso)flavonoids by liver enzymes was studied. These compounds can be extensively metabolized by phase I and II enzymes. A glucuronidation yield between 70-80% was observed. It was also shown that pyran and chain prenylation gave more complex hydroxylation patterns with 4 or more than 6 hydroxyl isomers, respectively, compared to unprenylated compounds (only 1 hydroxyl isomer).

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Chapter 1

General Introduction

Phenyl benzopyrans are secondary plant metabolites which can be found in *Leguminosae*. They comprise, amongst others, the subclasses flavonoids and isoflavonoids.[1] Their molecular structure bears similarity to that of the female hormone 17β-estradiol (E_2). As a result, many phenyl benzopyrans, so-called phytoestrogens, bind and activate the human estrogen receptors (hERs).[2] Upon consumption, this might lead to beneficial or adverse *in vivo* effects and is, for example, often correlated with decreased risk of diseases related to a Western lifestyle.[3-7] Dietary compounds with similar mode of action as E_2, like soy phenyl benzopyrans, can help to restore the estrogen level in postmenopausal women.[8,9] In this way, menopausal symptoms might be limited. Secondly, these compounds can help to lower the incidence of osteoporosis and the risk of hip fracture by maintaining or modestly improving bone mass. Compounds with a mode of action opposite to E_2, like ICI 182,780 and RU 58668, are well-known pharmaceuticals in treatment of patients with breast cancer.[10-12] Therefore, natural phytoestrogens might be important for the food and pharma industry, as food supplements and therapeutic agents, respectively. It is known that substitution of phenyl benzopyrans with five-carbon prenyl groups can modulate their estrogenic activities.[2] The molecular signatures underlying such modulation need to be established further. During and after absorption, metabolism of phenyl benzopyrans can occur, which influences the estrogenic activity.[13] To understand dietary effects, it is important to consider the metabolism of prenylated phenyl benzopyrans as well. The structure of phenyl benzopyrans, their estrogenicity and their metabolism will be discussed below.

PHENYL BENZOPYRANS

Structural characteristics of phenyl benzopyrans

Phenyl benzopyrans are plant-derived molecules, which are characterized by a C_6-C_3-C_6 carbon structure.[14] They can be divided into four classes based on the location of attachment of the aromatic B-ring on the benzopyran moiety: 2-phenyl benzopyrans, 3-phenyl benzopyrans, 4-phenyl benzopyrans and miscellaneous phenyl benzopyrans.[15] This thesis describes the estrogenic properties of 2-phenyl benzopyrans (flavonoids) and 3-phenyl benzopyrans (isoflavonoids) (Figure 1.1). Each class comprises various subclasses, amongst others flavones, flavanones, flavonols (all flavonoids), isoflavones, isoflavans and isoflavenes, pterocarpans (all isoflavonoids). They differ in the configuration of the C-ring or in the occurrence of an additional D-ring.[1] The IUPAC carbon numbering of the different compounds is also indicated in Figure 1.1. Compounds can be substituted with hydroxyl or methyl groups at different positions of the phenyl benzopyran backbone.

Figure 1.1. 2-Phenyl benzopyrans (flavonoids) and 3-phenyl benzopyrans (isoflavonoids) with their corresponding subclasses.

Glycosylation and prenylation of flavonoids and isoflavonoids

Flavonoids and isoflavonoids are mainly found in their glycosylated forms. The sugar moiety is predominantly connected via a hydroxyl group to form an *O*-glycoside.[14] A broad range of saccharides, including glucose and glucuronic acid, can be attached to flavonoids and isoflavonoids.

Prenylation of flavonoids and isoflavonoids refers to substitution with a C_5-isoprenoid (in this thesis referred to as prenyl), a C_{10}-isoprenoid (geranyl) or a C_{15}-isoprenoid (farnesyl) group. In this thesis, only the prenyl substitution is investigated.[16] Prenylated flavonoids and isoflavonoids are predominantly *C*-prenylated, with few reported *O*-prenylations.[16] Three different kinds of prenylation can occur: chain, pyran and furan prenylation (Figure 1.2). The most common chain prenylation is the addition of a 3,3-dimethyl allyl substituent (Figure 1.2A). A prenyl chain may undergo cyclisation with an *ortho*-phenolic hydroxyl group leading to a 6-membered pyran ring or a 5-membered furan ring. The common pyran substituent, 2,2-dimethylchromeno, is often referred to as pyran ring (Figure 1.2B). For furan substitutions different configurations are common, like 2”-(2-hydroxy-) isoprenyl furan (Figure 1.2C), 2”-isoprenyl furan (Figure 1.2D) and 2”-isoprenyl dihydrofuran (Figure 1.2E). Other prenyl configurations are possible[16], but the five configurations mentioned are studied in this thesis. Prenylation can occur at different positions on the flavonoid and isoflavonoid molecule. Possible prenylation positions are indicated with α, β, γ and δ. As the IUPAC carbon numbering of isoflavones differs from that of pterocarpans and pterocarpenes (Figure 1.1), we prefer to use a numbering system with Greek letters to indicate the position of prenylation (Figure 1.1). In this way, the similarities in position of prenylation are more clear than with the IUPAC numbering system.

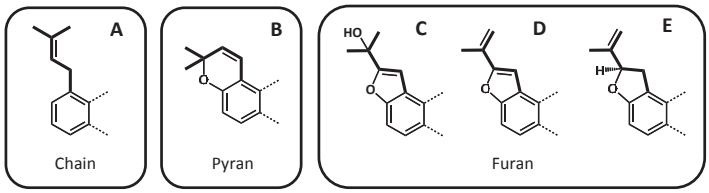

Figure 1.2. Overview of the most common prenyl substituents of flavonoids and isoflavonoids. 3,3-Dimethyl allyl (A), 2,2-dimethylchromeno (B), 2”-(2-hydroxy-) isoprenyl furan (C), 2”-isoprenyl furan (D) and 2”-isoprenyl dihydrofuran (E).

Analysis of flavonoids and isoflavonoids

Identification of flavonoids and isoflavonoids in plant extracts is mostly done by reversed-phase ultra-high-performance liquid chromatography (RP-UHPLC) coupled

to diode array detection (DAD) and/or mass spectrometry (MS).[15,17,18] DAD can be used for the detection of flavonoids and isoflavonoids, as it provides specific UV-vis spectral data for the different subclasses. Two bands can often be characterized: band I (300-350 nm) that is associated with absorption of the B-ring and band II (240-280 nm) that is associated with that of the A-ring.[19,20] Differences in intensity of the two bands are often caused by the kind of conjugated system. For example, the absorption maxima of 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes are both around 280 nm, whereas 6a,11a-pterocarpenes have a second absorption maximum between 330 and 364 nm due to their increased conjugated system. Besides the kind of conjugated system, additional oxygen atoms can have an influence on the spectral properties of a compound.[19,21]

Extra structural information can be obtained by mass spectrometry.[22] The most common techniques to ionize flavonoids and isoflavonoids are electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI).[23] Compounds can be analyzed in positive ion (PI) mode, by which the compound is protonated $[M+H]^+$, or in negative ion (NI) mode, by which the compound is deprotonated $[M-H]^-$. Fragmentation of molecular ions with, for example, collision-induced dissociation (CID) helps to obtain more structural information than the *m/z* ratio alone. In general, most fragment ions result from cleavage of the C-ring (*retro*-Diels-Alder (RDA) fragments), or are the result of neutral/radical losses. Cleavage of the C-ring can provide information about the substituents attached to the A- and B-ring in particular.[17] The nomenclature used for the RDA fragments is indicated by either $^{i,j}A^+$, $^{i,j}B^+$ and by $^{i,j}A^-$, $^{i,j}B^-$ for PI and NI mode fragmentation, respectively.[24] The superscripts i,j represent the bonds that are cleaved in the C-ring (Figure 1.3).

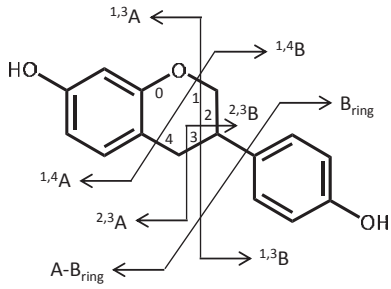

Figure 1.3. Nomenclature for RDA-fragmentation of flavonoids and isoflavonoids. The numbers represent the different bonds in the C-ring.

Neutral and radical losses are indicative for certain substituents. For example, a loss of 15 Da is often observed with a methyl group, and a loss of 176 Da is often observed when a glucuronic acid is attached to the compound.[25,26] Prenyl substituents can be

analyzed in detail because of their specific neutral losses in PI mode. The dominant neutral loss of 42 in PI mode is indicative for a pyran ring (2,2-dimethylchromeno), whereas a neutral loss of 56 in PI mode is indicative for a prenyl chain (3,3-dimethyl allyl).[27] Nevertheless, determination of the specific positions of the substituents with mass spectrometry is often difficult.

SOURCES OF FLAVONOIDS AND ISOFLAVONOIDS

Licorice

Licorice (*Glycyrrhiza glabra*) belongs to the *Leguminosae* family. Licorice roots are rich in prenylated isoflavans, isoflavenes isoflavones and flavanones.[27] The ethyl acetate extractable fraction of licorice roots contains prenylated flavonoids and isoflavonoids (Figure 1.4). Glabridin is the main representative, with a content between 0.08-0.35% (w/w) in dried roots.[28] In addition, glabrene, glabrone, glabrol and glabridin derivatives were found in licorice roots.[27]

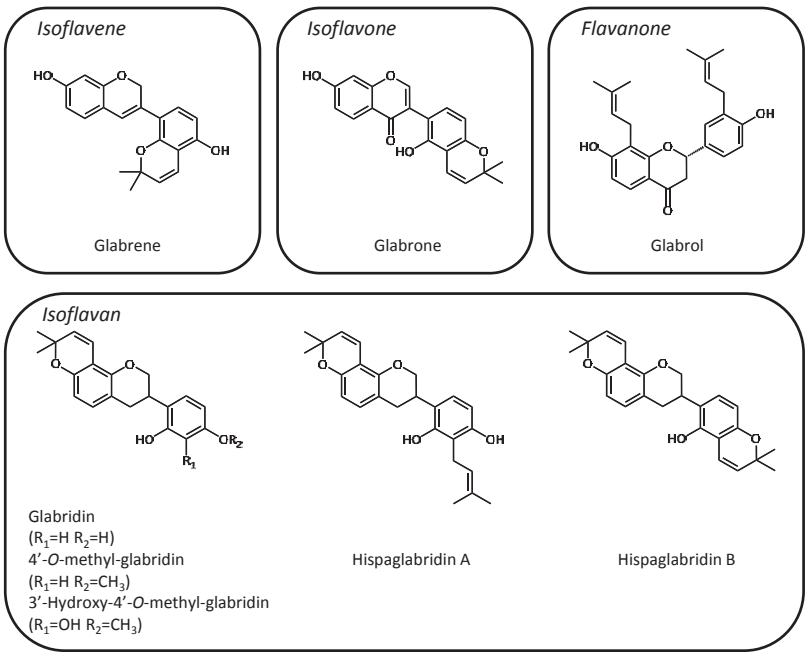

Figure 1.4. Molecular structures of the main prenylated flavonoids and isoflavonoids in licorice roots.

Soybean

Within the *Leguminosae* family, soybean (*Glycine max*) is a rich source of isoflavonoids. They comprise, amongst others, the subclasses of isoflavones, pterocarpans and coumestans.[14] Isoflavones are present in their glycosidic form (especially glucose and malonyl-glucose), whereas pterocarpans and coumestans occur in aglyconic form.[18,29] The content and structural diversity of isoflavonoids can be influenced by stimulation of the plant's defense system during germination, for example by wounding, fungal elicitors or light. The combination of germination, fungal elicitors and light can, for example, result in a 250-900% increase of the total isoflavonoid content, with a concomitant increase in structural diversity from one subclass only (isoflavones) in the seeds to a mixture of three subclasses (isoflavones, coumestans and 6a-hydroxy-pterocarpans) in elicited sprouts.[18,30] Along with the induction of additional subclasses, biotic stress also enhances prenylation of isoflavonoids. Deglycosylation of isoflavonoids often precedes prenylation. Prenylated 6a-hydroxy-pterocarpans, so-called glyceollins, are the main compounds accumulating in soybean sprouts, which are challenged with a combination of fungus and light.[18, 30] Nine prenylated 6a-hydroxy-pterocarpans and prenylated isoflavones, with prenylation on the A- or B-ring, have been annotated in soybean seedlings challenged by fungus.[30] Moreover, prenylation of coumestrol was also observed.[30] Figure 1.5 shows the prenylated isoflavonoids induced by stimulation of the plant's defense system, as this is the main focus of this thesis.

ESTROGENICITY

Human estrogen receptors (hERs)

The estrogen receptors are part of the nuclear hormone receptor superfamily. The main function of the ERs in human is the expression of genes essential for growth, sexual development and cell differentiation. They also play part in the central nervous system, cardiovascular system and regulation of bone formation.[31] Two human estrogen receptors exist, hERα and hERβ. Both subtypes consist of multiple domains, including the DNA–binding (DBD) and ligand-binding domain (LBD).[32] The DBD make the hERs recognize the estrogen response element (ERE) on the DNA, which is typically located in promoter sequences of estrogen-response genes.[33] As a consequence of binding of an activated receptor to an ERE, target gene expression will increase. The LBD of the receptor contains the site where compounds (the so-called ligands) are recognized and exists of 12 α-helices (H1-12) and 1 antiparallel β-sheet (B1 and B2) (Figure 1.6).

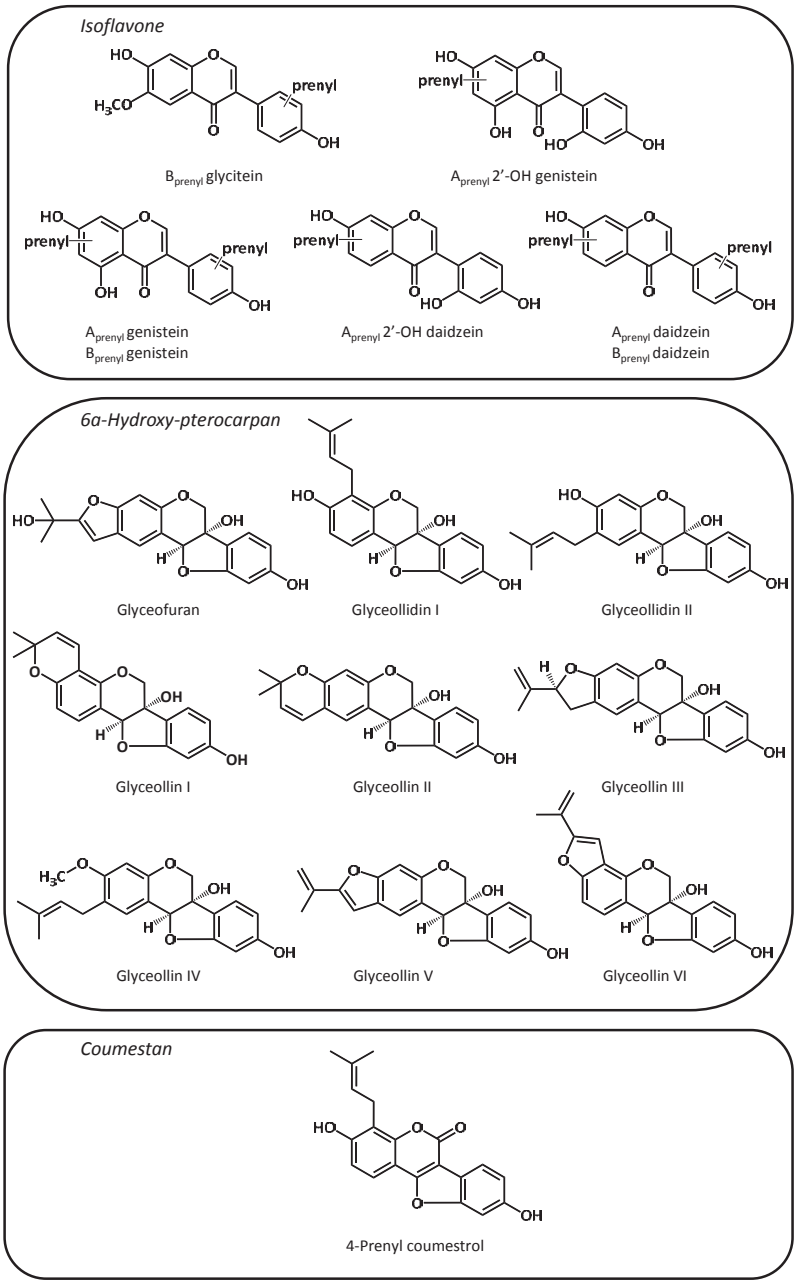

Figure 1.5. Molecular structures of the main prenylated isoflavonoids accumulating in soybean sprouts, which are challenged with a combination of fungus and light. Prenyl indicates that only one prenyl is attached to the A- or B-ring, as the exact position is unknown.

The LBD also houses the transcription activation function (AF2) region, which mainly consists of helix 12 together with some amino acids of helices 3, 4 and 5.[34] This region activates the receptor by recruiting coactivators and corepressors, this process is ligand-dependent. The other activation region amenable to the influence of co-regulators, AF1, is localized in a different domain and is ligand-independent.[35] The two ER subtypes have a large homology in the DBD (96%), but less in the LBD (53%). Based on these homologies it is predicted that not all ligands activate both hER receptors. Once activated, both receptors recognize and bind to the same ERE.[31,32,35]

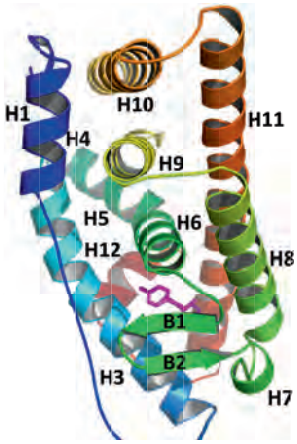

Figure 1.6. The overall configuration of ER-LBD. The different α-helices (H1-12) and 1 β-sheet (B1 and B2) are indicated. The full agonist diethylstilbestrol (DES) is shown in magenta.

Interaction with the human estrogen receptors

In order to bind to hERs and cause estrogenic responses, compounds need to have a chemical structure similar to the female sex hormone, E_2 (Figure 1.7). For flavonoids and isoflavonoids, a phenolic ring at one end and a hydroxyl group at the other end of the molecule are the most important characteristics for binding to the estrogen receptors. Optimal binding is achieved with two hydroxyl groups (comparable to positions 3 and 17 in E_2), although already one hydroxyl group suffices binding to the hERs.[36] Besides, the distance between the hydroxyl groups is critical and should resemble that between the 3-hydroxyl and the 17β-hydroxyl groups of E_2 (11 Å). Finally, high hydrophobicity and a lack of large polar groups in the core of the compound are important for binding to hERs.[35]

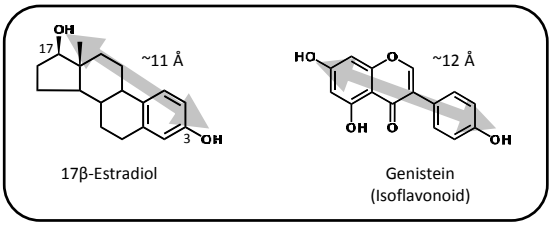

Figure 1.7. Molecular structures of 17β-estradiol and genistein. Length between the (most distant) hydroxyl groups is indicated with the arrow.

Upon binding of a compound, a conformational change of the receptor is evoked. In this way, two hERs can form a dimer and this dimer is able to specifically bind to ERE in the promoter region of the target genes. This system recruits co-regulators, which act as a bridge between the receptor and the transcription machinery on the DNA, the polymerase II complex (Figure 1.8).[33]

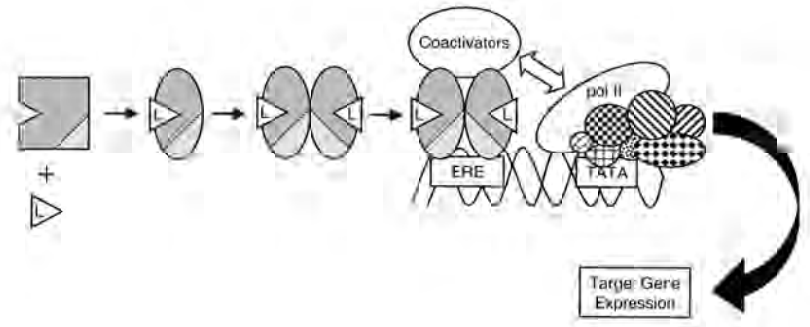

Figure 1.8. Model of the ER activation process.[33] ER is indicated by box or oval (indicating conformational changes in ER upon binding of a ligand) with LBD (dark grey) and DBD (light grey); L = ligand; ERE = estrogen response element; pol II = RNA polymerase II; TATA = TATA box.

Binding of compounds to hERs can result in an agonistic or an antagonistic mode of action.[2] The agonistic mode of action can be divided into full agonistic and partial agonistic. Full agonistic compounds show the same behavior as the natural ligand E_2, partial agonists also exhibit the same behavior as E_2, only their response is lower. However, it is also possible that a partial agonist has a mixed agonistic/antagonistic activity, the agonist activity being lower than that of E_2. In that case, both an agonistic activity and antagonistic activity is observed in the same assay. Compounds with an antagonistic mode of action have an action opposite to E_2 and can lower the signal of E_2.

Determination of estrogenic responses

Different assays exist that determine the estrogenic activity of compounds. Receptor ligand binding assays are used to determine if a compound can bind hERs. Transcriptional activity, and consequently agonistic and antagonistic mode of action, cannot be determined with these assays. The receptor ligand binding assays are based on competitive binding with labelled E_2, in which the fluorescence polarization signal of labelled E_2 decreases upon binding of the compound. To determine the activity of a compound, proliferation assays with mammalian cell lines and/or gene reporter assays with mammalian cell lines or yeast are used. Human breast cells (MCF-7), adenocarcinoma cells (Ishikawa) or kidney cells (HEK-293) are commonly used for assays with mammalian cell lines. Agonistic activity is measured by addition of the compound of interest alone. The concentration that yields a signal halfway between the baseline and the maximum response is called the effective concentration at half maximum response (EC_{50}). Agonistic activity can also be measured by co-supplementation of E_2 and the compound of interest. Co-supplementation is mostly done at the EC_{70} of E_2, *i.e.* the concentration corresponding to 70% of its maximum response. In both cases an increase in the signal should be observed (Figure 1.9). Equol is an example of a full agonist. In a similar way, antagonists and partial agonists can be identified. Partial agonistic compounds, for example genistein, will show a mixed agonistic and antagonistic activity (Figure 1.9), whereas antagonistic compounds, for example ICI 182,780, only show a decrease in the E_2 signal after co-supplementation and do not show agonistic activity (Figure 1.9). For comparison of the agonistic activities measured in different assays, relative estrogen potency (REP) values are used. REP values of a test compound are determined by dividing the EC_{50} of E_2 by the EC_{50} of the test compound. In this way, agonistic activities obtained in different assays can be compared.

Besides the different modes of action described above, compounds can also be ER subtype-selective or selective estrogen receptor modulating (SERMs).[2] ER subtype-selective compounds show a different mode of action towards different hERs in the similar assays. For example, *R,R*-THC shows an agonistic mode of action towards hERα and an antagonistic mode of action towards hERβ (Table 1.1).[37] However, ER subtype-selective compounds have also been correlated with compounds with a similar mode of action, but different affinity towards hERα and hERβ, tested in similar assays (Figure 1.9), *e.g.* compounds with an agonistic mode of action towards hERβ and (almost) no response towards hERα.[37] To determine subtype-selective behavior similar cells expressing only hERα and cells expressing only hERβ are needed.

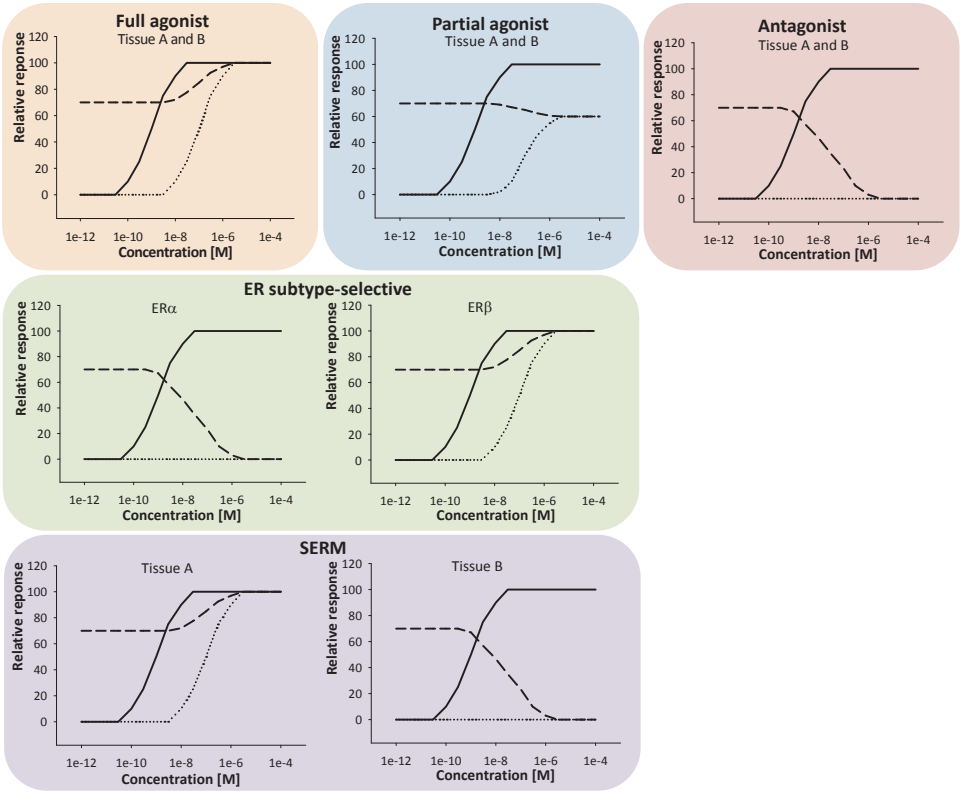

Figure 1.9. Schematic overview of the typical ER-response curves for different modes of estrogenic action. ——— = E_2, ····· = test compound, – – = test compound + E_2 (EC_{70}).

SERM behavior corresponds with a tissue-specific agonistic and antagonistic mode of action. Classification of SERMs can only be performed when compounds are studied in at least two different cell types. Researchers have classified tamoxifen as a direct antagonist due to its effects on breast cells[38], whereas later it became clear that tamoxifen has an agonistic mode of action on endometrial cells, and is thus actually a SERM[11]. SERM behavior can be caused by different cofactors/corepressors, abundance of receptor types (hERα vs hERβ), intracellular environments and intrinsic E_2 levels between the different tissues (Table 1.1). However, the exact mechanisms are still not fully understood.[2] To determine SERM behavior, the mode of action in different kinds of cell lines should be determined (Figure 1.9). In order to link SERM behavior to different intracellular environments and not to differences in abundance of the

receptor types, assays with different cell types should be used, each harboring the same hER, *e.g.* a hERα yeast bioassay and a hERα CALUX bioassay.

Conformational changes in hERs

The modes of action of the various ligands are characterized by conformational changes of the receptor (Figure 1.6), in which helix 12 plays a crucial role, as it is part of the transcription activation function (AF-2) region.[34] In the full agonistic mode, the conformational equilibrium of helix 12 favors the active conformation, resulting in the attraction of co-activators.[37] Some plasticity of the hERs upon binding of ligands has been described. For example, upon binding of *ortho*-trifluoromethylphenylvinyl estradiol, a hydrophobic pocket between helix 8 and the antiparallel β-sheet is formed. This is the result of the displacement of helix 7.[39] The displacement of helix 7 does not distort the AF-2 region, thereby resulting in the full agonistic response. Partial agonism occurs when a compound slightly influences the orientation of helix 11. Consequently, the compound is incapable of shifting the conformational equilibrium of helix 12 to the fully active conformation. Antagonistic compounds shift the conformational equilibrium of helix 12 to an inactive conformation in two possible ways: direct and indirect. Direct antagonists influence the position helix 12 directly by steric hindrance. Indirect antagonists influence the position of helix 12 by interfering with other helices of the receptor, like helices 3 or 11, or by inappropriate contacts with the binding cavity[35,37,40], resulting in an impaired recruitment of co-activators. It is impossible to distinguish between direct and indirect antagonists by transcriptional activation assays. For this, the crystal structures with the respective compounds should be determined. The correlations between the assay response, receptor conformation and the final classification of the compounds are illustrated, with a few examples, in Table 1.1. Besides, the classification of SERMs and ER subtype-selectivity is shown (Table 1.1). It should be noted that the classification of ER subtype-selectivity is debatable and we will further elaborate on this in the **General Discussion**.

Table 1.1. Classification of compounds with estrogenic behavior.

I. Response to an individual receptor: agonistic or antagonistic mode of action			
Representative compound	*Assay response*	*Conformation receptor*	*Classification*
17β-Estradiol	Solely agonist (response of 100%)	Normal conformation	Full agonist
Genistein	Solely agonist (response <100%) *or* Mixed agonist + antagonist	Helix 11 slightly modulated	Partial agonist
ICI 182,780	Solely antagonist	Helix 12 modulated	Direct antagonist
R,R-THC	Solely antagonist	Helices other than helix 12 modulated	Indirect antagonist
II. Response to hERα and hERβ in same environment: ER subtype-selectivity			
R,R-THC	- Different mode of action towards hERα and hERβ - (Different affinity, but the same mode of action towards hERα and hERβ)[a]		
III. Response to hER in different environments (tissues): SERM behavior			
Tamoxifen	Different modes of action in different environments/tissues/assays due to variation in: - Presence of cofactors/corepressors - Intrinsic E_2 level - Abundance of receptor types (hERα vs hERβ)		

[a] This classification of ER subtype-selectivity is debatable.

Modulation of estrogenic responses of flavonoids and isoflavonoids

Flavonoids and isoflavonoids can exhibit estrogenic activity (Table 1.2), as they bear structural similarity to E_2. Most unprenylated flavonoids and isoflavonoids show an agonistic activity, which can be modulated by prenyl substitution.[2,16,41] Prenylation of flavones, flavanones and flavonols has been linked to an increased affinity towards hERα.[42-44] For example, chain prenylation at the 8-position of the flavanone naringenin resulted in an increased agonistic activity.[45,46] The isoflavonoid kwakhurin has an additional prenyl chain, hydroxyl and methyl group compared to daidzein, which results in a stronger agonistic activity of kwakhurin compared to daidzein.[47] This is probably caused by the additional chain prenyl, but it cannot be excluded that the additional hydroxyl and methyl groups also influence the activity. On the other hand, chain prenylation of the isoflavone genistein resulted in a decrease of agonistic activity.[45] This indicates that chain prenylation can change the activity towards the hERs, but does not change the mode of action, as compounds remain agonistic. However, it has been suggested that pyran prenylation of isoflavonoids can change the mode of action from agonistic to antagonistic, especially when the pyran prenyl is attached to the A-ring, α- or β-position (Figure 1.1).[2,48]

Table 1.2. Overview of mode of estrogenic action of unprenylated and prenylated flavonoids and isoflavonoids.

Compound	Molecular characteristics				Estrogenic characteristics				Ref.
					Human cell line[a]		Yeast R		
	1st prenyl	Pos.	2nd prenyl	Pos.	Type	Action	Action (type hER)	Overall mode of action	
Coumestans									
Unprenylated									
Coumestrol	-	-	-	-	Ishikawa[q] P	Agonist	Agonist (α)	Agonist	*11, 59*
Wedelolactone	-	-	-	-	MCF-7 P; T47D P	Agonist		Agonist	*60*
Prenylated									
Glycyrol	Chain[b]	β	-	-	RLBA	Estrogenic	-	Estrogenic[c,d,e]	*51*
Isoflavanones									
Unprenylated									
Derivatives of 7,4'-diOH isoflavanone	-	-	-	-	MCF-7 P+R; T47D P+R	Agonist	-	Agonist[c]	*61, 62*
Dihydrodaidzein	-	-	-	-	MCF-7 P; HEK-293 R	Agonist	No agonist[c] (α + β)	Agonist[c]	*63, 64*
Dihydrogenistein	-	-	-	-	MCF-7 P; HEK-293 R	Agonist	-	Agonist[c]	*64*
Prenylated									
Kievitone	Chain[b]	α	-	-	MCF-7 P+R; HEK-293 R	Agonist and antagonist	-	Partial agonist[r]	*65*
Isoflavans									
Unprenylated									
5'-*O*-Me vestitol	-	-	-	-	MCF-7 P+R; T47D P+R	Agonist	-	Agonist[c]	*61*
Equol	-	-	-	-	MCF-7 P; Ishikawa[q] P	Agonist	Agonist (α)	Agonist	*11, 59, 66*
Mucronulatol	-	-	-	-	MCF-7 P+R; T47D P+R	Agonist	-	Agonist[c]	*61*
Vestitol	-	-	-	-	MCF-7 P+R; T47D P+R	Agonist	-	Agonist[c]	*61*
Prenylated									
2'-*O*-Me Glabridin	Pyran[f]	α	-	-	MCF-7 P; T47D P	Agonist	-	Agonist[c]	*67*
4'-*O*-Me Glabridin	Pyran[f]	α	-	-	MCF-7 P; T47D P	Agonist	-	Agonist[c]	*67*
2',4'-*O*-Dime Glabridin	Pyran[f]	α	-	-	MCF-7 P; T47D P	Agonist	-	Agonist[c]	*67*
Glabridin	Pyran[f]	α	-	-	MCF-7 P+R; T47D P+R	Agonist	Antagonist (α)	phytoSERM	*50, 67, 68*
Tetrahydroglabrene	Pyran[g]	δ	-	-	RLBA	Estrogenic	-	Estrogenic[c,e]	*51*
Hispaglabridin A	Pyran[f]	α	Chain[b]	δ	RLBA	Estrogenic	-	Estrogenic[c,e]	*68*
Hispaglabridin B	Pyran[f]	α	Pyran[f]	δ	RLBA	Estrogenic	-	Estrogenic[c,e]	*68*

Isoflavenes									
Unprenylated									
Dehydroequol	-	-	-	-	HEC-1 R	Agonist	-	Agonist	*69*
Prenylated									
Glabrene	Pyran[f]	δ	-	-	MCF-7 P+R; T47D P+R; RLBA	Agonist	Agonist[m] (α + β)	Agonist	*50, 51, 68*
Isoflavones									
Unprenylated									
Biochanin A	-	-	-	-	Ishikawa[q] R ; MCF-7 P	Agonist	Agonist (α + β)	Agonist	*44, 70, 71*
Daidzein	-	-	-	-	Ishikawa[q] P	Agonist	Agonist (α)	Agonist	*48, 59*
Formononetin	-	-	-	-	Ishikawa[q] R	Agonist	Agonist (α + β)	Agonist	*70, 71*
Genistein	-	-	-	-	Ishikawa[q] P; MCF-7 P+R	Agonist	Agonist (α)	Agonist	*11, 44, 45, 48, 59*
Glycitein	-	-	-	-	MCF-7 P	No Agonist[c]	Agonist (α + β)	Agonist	*63*
Prenylated									
8-prenylformononetin	Chain[b]	α	-	-	MCF-7 P+R	No agonist	-	Not estrogenic[c]	*72*
8-prenylgenistein (8DMAG)	Chain[h]	α	-	-	MCF-7 P	Partial agonist[r]	Antagonist (α)	phytoSERM	*44*
8-prenylgenistein (8PG)	Chain[b]	α	-	-	Ishikawa[q] R; MCF-7 R; UMR-106 P; MCF-7 P	Agonist No agonist[c]	No agonist[c] (α)	Agonist	*42, 45, 73*
6-prenylgenistein (6DMAG)	Chain[h]	β	-	-	Ishikawa[q] R; MCF-7 R	Agonist	No agonist[c] (α)	Agonist	*45*
6-prenylgenistein (6PG)	Chain[b]	β	-	-	UMR-106 P	n.d.[l]	n.d.[l] (α)	n.d.[l]	*48, 73*
Kwakhurin	Chain[b]	γ			MCF-7 P	Agonist	-	Agonist[c]	*47*
Isopoegin B	Chain[b]	δ			U2OS R	Agonist	-	Agonist[c]	*52*
7,8-(2,2-diMe-pyrano) daidzein	Pyran[f]	α	-	-	MCF-7 R	Antagonist	-	Antagonist	*49*
Alpinumisoflavone	Pyran[f]	β	-	-	-	-	No agonist; no antagonist (α)	Not estrogenic	*48*
Corylin	Pyran[f]	δ	-	-	UMR-106 P	Agonist	-	Agonist[c]	*74*
Isopoegin D	Pyran[j]	δ	-	-	U2OS R	Agonist	-	Agonist[c]	*52*
6,8-diprenyl 3'OH genistein	Chain[b]	α	Chain[b]	β	MCF-7 P	Antagonist	Antagonist (α)	Antagonist	*44, 48*
6,8-diprenyl-genistein	Chain[b]	α	Chain[b]	β	UMR-106 P	Agonist	-	Agonist[c]	*73*
Erysenegalensein E	Chain[k]	α	Chain[b]	β	-	-	No agonist; no antagonist (α)	Not estrogenic	*48*
Isoerysenegalensein E	Chain[b]	α	Chain[k]	β	-	-	Antagonist (α)	Antagonist	*48*
Millewanin G	Chain[b]	α	Chain[k]	β	-	-	Antagonist (α)	Antagonist	*56*

Millewanin H	Chain[k]	α	Chain[b]	β	-	-	Antagonist (α)	Antagonist	*56*
Auriculasin	Chain[b]	α	Pyran[f]	β	MCF-7 P	Antagonist	Antagonist (α)	Antagonist	*44, 48*
Warangalone	Chain[b]	α	Pyran[f]	β	-	-	Antagonist (α)	Antagonist	*48*
Furowanin A	Chain[b]	α	Furan[l]	β	-	-	Antagonist (α)	Antagonist	*48*
Furowanin B	Furan[l]	α	Chain[b]	β	-	-	Antagonist (α)	Antagonist	*56*
Isolupalbigenin	Chain[b]	α	Chain[b]	δ	U2OS R	Agonist	-	Agonist[c]	*52*
2',5'-diprenyl 3'OH genistein	Chain[b]	γ	Chain[b]	δ	MCF-7 P	Antagonist	Antagonist (α)	Antagonist	*44*
2'-prenyl 4',5'-(2-2-diMe-pyrano) 3'-OH genistein	Chain[b]	γ	Pyran[f]	δ	MCF-7 P	Antagonist	Antagonist (α)	Antagonist	*44*
Pterocarpans									
Unprenylated									
Glycinol	-	-	-	-	MCF-7 R; HEK-293 R	Agonist	-	Agonist	*75*
Maackiain	-	-	-	-	RLBA	No binding	-	Not estrogenic[c]	*76*
Medicarpin	-	-	-	-	MCF-7 P COS-7 R	Antagonist Agonist	Agonist (α + β)	PhytoSERM	*77, 78*
Pisatin	-	-	-	-	RLBA	Estrogenic	-	Estrogenic[c,e]	*79*
Prenylated									
Bitucarpin A	Chain[b]	α	-	-	-	-	Antagonist (α)	Antagonist	*43*
Puemircarpene	Chain[b]	γ	-	-	MCF-7 P	No agonist	-	Not estrogenic[c]	*47*
Glyceollin III	Furan[p]	β	-	-	MCF-7 R MCF-7 P	Antagonist[o] Agonist[n]	-	phytoSERM	*53, 55*
(-)-Glyceollin I	Pyran[f]	α	-	-	MCF-7 R; HEK-293 R MCF-7 P	Antagonist Agonist[n]	-	phytoSERM	*53-55*
Glyceollin II	Pyran[f]	β	-	-	MCF-7 R MCF-7 P	Antagonist[o] Agonist[n]	-	phytoSERM	*53,55*
Phaseollin	Pyran[f]	δ	-	-	MCF-7 P+R; HEK-293 R	Partial agonist[r]	-	Partial agonist	*65*
(+)-Tubersin	Pyran[f]	δ	-	-	MCF-7 P	No agonist	-	Not estrogenic[c]	*47*
Erybraedin C	Chain[b]	α	Chain[b]	δ	-	-	n.d.[i] (α)	n.d.[i]	*43*
Flavanones									
Unprenylated									
Liquiritigenin	-	-	-	-	MCF-7 P	Agonist	Agonist (α)	Agonist	*44*
Naringenin	-	-	-	-	Ishikawa[q] R ; MCF-7 P+R	Agonist	Agonist (α + β)	Agonist	*44, 45, 80*
Prenylated									
8-prenylnaringenin (8PN)	Chain[b]	α	-	-	Ishikawa[q] R ; MCF-7 P+R	Agonist	Agonist (α)	Agonist	*42, 45*

6-prenylnaringenin (6DMAN)	Chain[h]	β	-	-	Ishikawa[q] R ; MCF-7 R	Agonist	Agonist (α)	Agonist	*45*
6-prenylnaringenin (6PN)	Chain[b]	β	-	-	MCF-7 P	No agonist[c]	-	Not estrogenic[c]	*42*
6,8-diprenylnaringenin	Chain[b]	α	Chain[b]	β	MCF-7 P	No agonist[c]	-	Not estrogenic[c]	*42*
Flemiphilippinin D	Chain[b]	α	Chain[b]	β	MCF-7 P	No agonist; no antagonist	Antagonist (α)	Antagonist	*44*
Flemichin D	Chain[b]	α	Pyran[f]	β	MCF-7 P	Antagonist	Antagonist (α)	Antagonist	*44*
Flavones									
Unprenylated									
3-hydroxyflavone	-	-	-	-	MCF-7 P	Antagonist	No agonist[c] (α)	Antagonist	*81*
5-hydroxyflavone	-	-	-	-	MCF-7 P	No agonist; no antagonist	No agonist[c] (α)	Not estrogenic[c]	*81*
7-hydroxyflavone	-	-	-	-	MCF-7 P	No agonist; no antagonist	No agonist[c] (α)	Not estrogenic[c]	*81*
Chrysin	-	-	-	-	MCF-7 P	Antagonist	No agonist[c] (α)	Antagonist	*81*
Fisetin	-	-	-	-	MCF-7 P	Agonist	No agonist[c] (α)	Agonist	*81*
Flavone	-	-	-	-	MCF-7 P	No agonist; no antagonist	No agonist[c] (α)	Not estrogenic[c]	*81*
Galangin	-	-	-	-	MCF-7 P	Agonist	No agonist[c] (α)	Agonist	*81*
Kaempferol	-	-	-	-	MCF-7 P	Agonist	Agonist (α)	Agonist	*81*
Luteolin	-	-	-	-	MCF-7 P	Agonist	Antagonist (α)	phytoSERM	*43, 81*
Prenylated									
8-prenylapigenin	Chain[b]	α	-	-	MCF-7 P	Agonist	-	Agonist	*42*
8-prenylquercetin	Chain[b]	α	-	-	MCF-7 P	Agonist	-	Agonist	*42*
Licoflavone C	Chain[b]	α			-	-	Partial agonist[r] (α)	Partial agonist	*43*

P, proliferation assay; R, gene reporter assay; RLBA, receptor ligand-binding assay; [a] Can contain hERα and hERβ, depending on the cell line used; [b] 3,3-dimethyl allyl; [c] Antagonistic activity not determined; [d] >500 times weaker binding than coumestrol; [e] Estrogenicity only measured by ER-binding; [f] 2,2-dimethyl chromeno; [g] 2,2-dimethyl chromano; [h] 1,1-dimethyl allyl; [i] Not determined in assay due to cytotoxicity; [j] 2,2-dimethyl-3-hydroxy chromeno; [k] 2-hydroxy-3-methylbut-3-enyl; [l] 2"-(2-hydroxy-)isopropenyl dihydrofuran; [m] Glabrene was tested as a purified fraction and should be tested in pure form to accurately determine its estrogenic activity; [n] Measured as a mixture of glyceollin I, II and III; [o] Glyceollins II and III might be considered as partial antagonists; [p] 2"-isopropenyl dihydrofuran; [q] Ishikawa cells are based on an adenocarcinoma cell line. [r] Classified as partial agonist, as the compound has agonistic and antagonistic activity in the same assay.

For example, pyran prenylation of daidzein led to an antagonistic mode of action for 7,8-(2,2-diMe-pyrano)daidzein.[49] Moreover, pyran prenylation and hydroxylation of equol modulated the mode of action from full agonistic towards antagonistic in an hERα yeast bioassay.[50] However, pyran prenylation does not always lead to an antagonistic mode of action. Compounds with pyran prenylation, especially on the B-ring at the δ-position (Figure 1.1), can still have an agonistic mode of action, for example glabrene and isopoegin D.[51,52] SERM behavior has been suggested for the pyran and furan prenylated glyceollins I, II and III. They are known to have an antagonistic mode of action in MCF-7 cells and they inhibit estrogen-induced tumor progression.[53,54] However, they have an agonistic mode of action in a MCF-7 based proliferation assay (E-screen).[55] Double prenylation of flavonoids and isoflavonoids mainly resulted in an antagonistic mode of action.[56]

An overview of estrogenic activity of different flavonoids and isoflavonoids is shown in Table 1.2. ER subtype-selective compounds are not taken into account, as cell lines can contain both hERα and hERβ and it is known that the ratio between hERα and hERβ can influence the total activity of a compound.[57] Moreover, most flavonoids and isoflavonoids are only tested on hERα, and not on hERβ, which makes it impossible to determine ER subtype-selectivity. SERM behavior indicated in Table 1.2 is probably only caused by differences in intracellular environments and not due to differences in abundance between the receptor types (Table 1.1), as all compounds were tested on hERα in a yeast bioassay and in MCF-7 cell lines, which mainly contained hERα.[58] In conclusion, evidence is accumulating that prenylation modulates estrogenic activity, but the exact structure-activity relationships need to be established further.

METABOLISM

It is known that flavonoids and isoflavonoids can be absorbed in the small intestine after ingestion.[82-84] Compounds can be modified during and after absorption by enterocytes and liver.[85] Metabolism can be divided into two phases. Phase I metabolism leads to hydroxylation, epoxidation, hydrogenation, demethylation, hydrolysis of the sugar moiety and isomerization.[86-88] Different enzymes play a role during phase I metabolism, but cytochrome P450 enzymes (CYP) are the most important ones (Figure 1.10). The other enzymes, that contribute, albeit to a minor extent, are reductases, hydrolases and isomerases. Phase II metabolism consists of conjugation reactions with glucuronate, sulfate, acetyl, glutathione or methyl groups. These reactions are facilitated by UDP-glucuronosyl transferases (UGT), sulfotransferases (SULT), N-acetyl transferases (NAT), glutathione S-transferases (GST) and methyl transferases (MET)[89], respectively (Figure 1.10). Metabolism is

performed to facilitate excretion via the kidneys.[90] Addition of hydroxyl groups during phase I metabolism is not very efficient in making a compound more polar.[91] Nevertheless, hydroxylation during phase I facilitates phase II metabolism. Therefore, phase I often precedes phase II metabolism, although not necessarily so.[92]

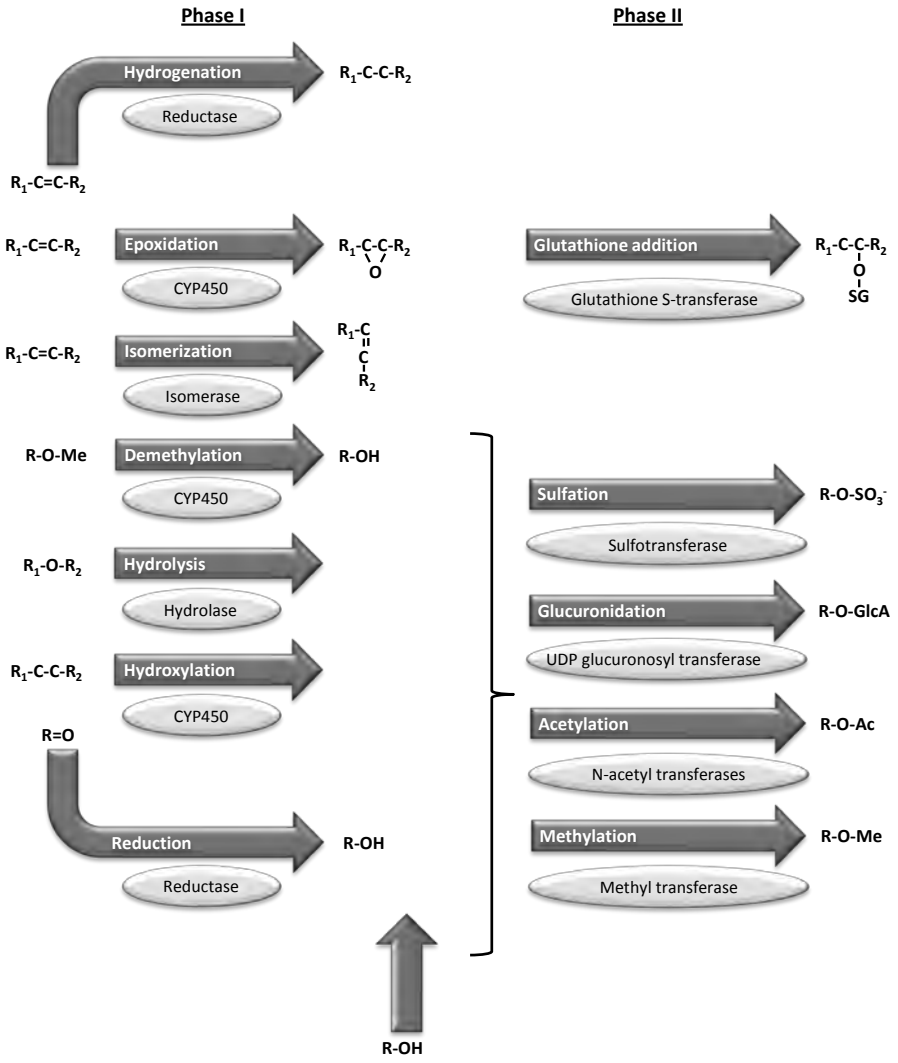

Figure 1.10. Phase I and phase II metabolic pathways. Enzymes are indicated below the different reactions.

Metabolism can be studied in different ways, from simplified *in vitro* systems, like microsomes, S9 mixtures and cell lines to *in vivo* animal or human models.[93] Microsomes consist of vesicles of the hepatocyte endoplasmic reticulum and contain almost only CYP and UGT enzymes.[93] The S9 mixture contains microsomes and the cytosolic fraction. The latter contains water-soluble components of the cytoplasm, including the soluble phase II enzymes GST and SULT. The S9 mixture contains all phase I and phase II enzymes. *In vivo* studies have the advantage that they resemble the *in vivo* situation better than *in vitro* systems. *In vitro* systems, on the other hand, are in general easier in use and ethically better accepted. Another advantage of *in vitro* assays with S9 mixtures is the possibility to determine metabolites formed in the different phases. This is achieved by adding different co-factors. In this way, an improved insight in the different phases of metabolism can be obtained.

Most studies analyzing metabolites of flavonoids and isoflavonoids focus on *in vitro* liver metabolism (microsomes) or on *in vivo* metabolism (Table 1.3). Metabolism of aglyconic isoflavones, like genistein, daidzein, glycitein, biochanin A and formononetin, has been shown to result in hydroxylation and glucuronidation. For most unprenylated compounds glucuronidation and sulfation are the major metabolic reactions. For the double chain prenylated isoangustone A, hydroxylation besides glucuronidation and sulfation, seems also of importance.[87] Moreover, it has been shown that *in vivo* rat metabolism of prenylated pterocarpans from soybean resulted in hydroxylation, epoxidation, glutathionylation, glucuronidation and sulfation.[94,95] This suggests that during metabolism hydroxylation is more important for prenylated compounds than for unprenylated compounds. Besides the attachment of different polar groups to the prenylated pterocarpans, also epoxidation of the prenyl double bond has been observed.[94] Epoxidation of the double bond of the prenyl chain was also observed with hop flavonoids using human liver microsomes, ultimately leading to pyran and furan derivatives.[96,97] The exact positions of the substituents were mostly not determined. In conclusion, detailed structure-metabolism relationships are still lacking, as systematic studies to determine differences in metabolism between flavonoids and isoflavonoids with different backbone structure and different kind/number of prenylation have not yet been carried out.

Table 1.3. Overview of the metabolites formed during metabolism of flavonoids and isoflavonoids.

Compound	Assay	Origin	Phase I	Phase II	Ref.
Isoflavans					
Unprenylated					
Equol	*In vivo*	Human	-	Glucuronidation, sulfation	*98*
	In vitro liver microsomes	Human	Hydroxylation	n.d.	*99*
Prenylated					
Glabridin	*In vitro* liver microsomes	Human	n.d.	Glucuronidation, glutathionylation[a]	*100, 101*
Isoflavones					
Unprenylated					
Genistein	*In vitro* liver microsomes	Human	Hydroxylation	Glucuronidation	*102, 103*
	In vitro SULT enzymes	Human	n.d.	Sulfation	*103*
	In vivo	Human	Hydroxylation	Glucuronidation, sulfation	*102, 103*
Daidzein	*In vitro* liver microsomes	Human	Hydroxylation	Glucuronidation	*102, 103*
	In vivo	Human	Hydroxylation	Glucuronidation, sulfation	*102, 103*
Prenylated					
Isoangustone A	*In vivo*	Rat	Hydroxylation	Glucuronidation	*87*
Pterocarpans					
Prenylated					
Glyceollin III, II, I	*In vivo*	Rat	Epoxidation, hydroxylation, saturation double bond	Glucuronidation, sulfation, glutathionylation	*94, 95*
	In vitro caco-2 cells	Human	Hydroxylation	Sulfation	*104*
Flavanones					
Unprenylated					
Naringenin	*In vitro* liver microsomes	Rat	Hydroxylation	Glucuronidation	*105, 106*
Liquiritin apioside	*In vivo*	Rat	Hydrolysis sugar moiety, isomerization	Sulfation	*87*
Liquiritin	*In vivo*	Rat	Hydrolysis sugar moiety, isomerization	Glucuronidation	*87*
Liquiritingenin	*In vivo*	Rat	Isomerization	Glucuronidation, sulfation	*87*
Prenylated					
8-Prenylnaringenin	*In vitro* liver microsomes	Human	Epoxidation, hydroxylation	n.d.	*96*
Isoxanthohumol	*In vitro* liver microsomes	Human	Epoxidation, hydroxylation, demethylation	n.d.	*97*
Flavones					
Unprenylated					
Dihydroxyflavone	*In vivo*	Rat	-	Glucuronidation	*87*

[a] not formed by GST, but formed after reaction with electrophilic metabolites generated by CYPs. n.d. not determined. – not detected.

AIM AND OUTLINE OF THE THESIS

As mentioned above, (prenylated) flavonoids and isoflavonoids from natural sources can be used as therapeutic agent, as they might have potentially beneficial or adverse effects *in vivo*. These effects are often mediated through interaction with hERs. Evidence is accumulating that unprenylated flavonoids and isoflavonoids show mainly an agonistic mode of action and that prenylation can modulate this mode of action. However, structure-activity relationships need to be established further. For this, flavonoids and isoflavonoids with different backbone structure and different kind/number of prenylation need to be tested. In this thesis, it is hypothesized that especially double prenylation leads to an antagonistic response, as most double prenylated compounds showed an antagonistic mode of action. It is also hypothesized that pyran prenylation on the δ position of isoflavonoids will not change the agonistic mode of action, whereas pyran prenylation on the α and β positions seems to be more efficient in changing the mode of action. The first aim of this thesis is to understand structure-activity relationships of prenylated flavonoids and isoflavonoids towards the hERs. Flavonoids and isoflavonoids are metabolized after and during absorption, thereby changing their molecular structure. For *in vivo* bioactivity, it is important to understand their metabolism. From the above it is clear that prenylated (iso)flavonoids are more extensively hydroxylated than their unprenylated look-alikes. So, in this thesis it is hypothesized that prenylation increases hydroxylation and that hydroxylation mainly occurs on the prenyl groups. The second aim of this thesis is to understand structure-metabolism relationships of prenylated flavonoids and isoflavonoids.

In this thesis purification of prenylated flavonoids and isoflavonoids is performed in order to establish the structure-activity or structure-metabolism relationships. We observed that heat and acid could influence the composition of 6a-hydroxy-pterocarpans from an extract of challenged soybean sprouts during purification. Therefore, the influence of heat and acid on the composition and estrogenicity of 6a-hydroxy-pterocarpans is studied (**Chapter 2**). In **Chapter 3**, the estrogenic activity of unprenylated and prenylated flavonoids and isoflavonoids from licorice was determined with a yeast bioassay. In addition, the estrogenic responses were correlated to the structural features of the receptors. In **Chapter 4**, the estrogenic activity of prenylated isoflavonoids was determined in two different assays in order to determine if compounds from elicited soybean sprouts exhibit SERM behavior. Secondly, structural characteristics needed for ER subtype-selective or SERM behavior were discussed. In **Chapter 5**, *in vitro* liver metabolism of unprenylated and prenylated flavonoids and isoflavonoids from licorice was studied. The most

important outcomes of this research are discussed in **Chapter 6**, including structural characteristics that modulate estrogenicity. The estrogenic activity of the phytoestrogens studied in this research are compared with known (synthetic) estrogenic compounds. Also, the activity of flavonoids and isoflavonoids towards other receptors and the possible influences of metabolism on the estrogenic activity are dealt with.

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Chapter 2

Structural changes of 6a-hydroxy-pterocarpans upon heating modulate their estrogenicity

ABSTRACT

The isoflavonoid composition of an ethanolic extract of fungus-treated soybean sprouts was strongly altered by a combined acid/heat treatment. UHPLC-MS analysis showed that 6a-hydroxy-pterocarpans were completely converted into their respective, more stable, 6a,11a-pterocarpenes, whereas other isoflavonoids, from the isoflavone and coumestan subclasses, were affected to a much lesser extent (loss of ~15%). Subsequently, mixtures enriched in prenylated 6a-hydroxy-pterocarpans (pools of glyceollin I/II/III and glyceollin IV/VI) or prenylated 6a,11a-pterocarpenes (pools of dehydroglyceollin I/II/III and dehydroglyceollin IV/VI) were purified, and tested for activity on both human estrogen receptors (hERα and hERβ). In particular, the mode of action towards hERα changed, from agonistic for glyceollins to antagonistic for dehydroglyceollins. Towards hERβ a decrease in agonistic activity was observed. These results indicate that the introduction of a double bond with the concomitant loss of a hydroxyl group in 6a-hydroxy-pterocarpans extensively modulates their estrogenicity.

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INTRODUCTION

Isoflavonoids (3-phenyl benzopyrans) are phenolic compounds that mainly occur in *Leguminosae*. They comprise, amongst others, the subclasses of isoflavones, pterocarpans, pterocarpenes and coumestans.[3] The molecular structure of isoflavonoids bears similarity to the female hormone 17β-estradiol. As a result many isoflavonoids bind and activate the human estrogen receptors (ERs). This results in an agonistic, an antagonistic or a tissue-specific agonistic/antagonistic response (selective estrogen receptor modulators: SERMs).[4] Dietary intake of such, so-called phytoestrogens might lead to *in vivo* beneficial effects, although some adverse effects, like genotoxicity, are observed.[5] Nevertheless, *Leguminous* extracts containing these compounds might be used as food supplement or as therapeutic agent.[6,7]

Within the *Leguminosae* family, soybean (*Glycine max*) is a rich source of isoflavonoids. The content and structural diversity of isoflavonoids can be influenced by stimulation of the plant's defense system during germination, for example by wounding, fungal elicitors or light. The combination of germination, fungal elicitors and light can, for example, result in an isoflavonoid increase of 250% and an increase in structural diversity from only isoflavones in the seeds to a mixture of isoflavones, coumestans and 6a-hydroxy-pterocarpans in elicited sprouts.[1] Along with the induction of additional subclasses, biotic stress also enhances prenylation of isoflavonoids. Prenylated 6a-hydroxy-pterocarpans, so-called glyceollins, are the main compounds accumulating in fungus- and light-challenged soybean sprouts (Figure 2.1).[1,2]

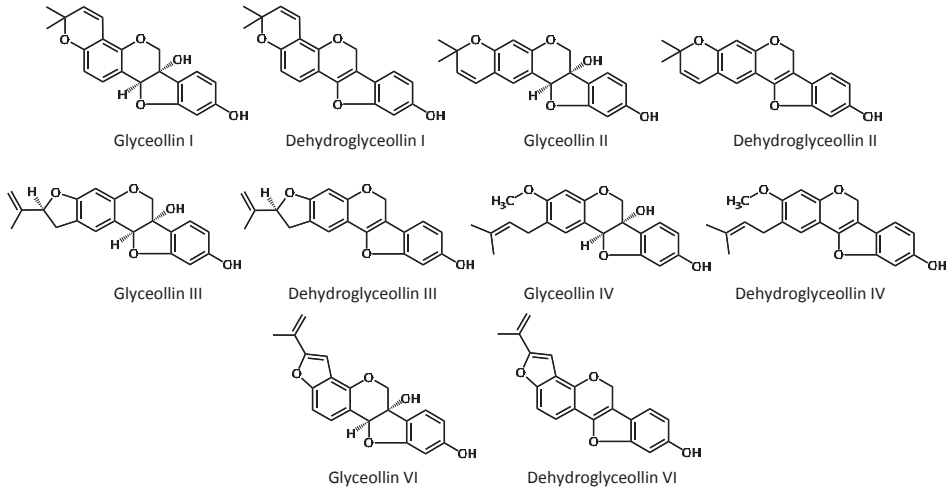

Figure 2.1. Structures of glyceollins and dehydroglyceollins assigned in soybean extracts.

Glyceollin I is known as an ER antagonist and inhibits estrogen-induced tumor progression in MCF-7 cells.[8,9] Glyceollins II and III also show ER an antagonistic mode of action, although less pronounced. However, in another study[10] it was shown that a glyceollin mixture exhibits an agonistic mode of action towards the estrogen receptor. Taken together, these data imply that glyceollins might act as selective estrogen receptor modulators (SERMs).

In order to purify or to process (mixtures of) 6a-hydroxy-pterocarpans, it is important to know the stability of these isoflavonoids. It has been suggested that the 6a-hydroxy-pterocarpan, glyceollin I, is unstable at pH's below 4 and at temperatures above 40 °C. Although the structural characteristics of the degradation products were not revealed, it was proposed[11] that glyceollin I might lose the hydroxyl group at the 6a position of the molecule (dehydration) to form the thermodynamically more stable pterocarpene dehydroglyceollin I. Hence, it is clear that processing can change the isoflavonoid profile and consequently change the bioactivity of isoflavonoid preparations.

In the present study, heat and acid (100 °C; 0.56 M HOAc) were used to convert 6a-hydroxy-pterocarpans into products, which were characterized as 6a,11a-pterocarpenes. The structural characteristics of these two subclasses are different, which might affect their estrogenic properties. In order to test this hypothesis, fractions enriched in 6a-hydroxy-pterocarpans or 6a,11a-pterocarpenes were obtained and their estrogenic properties were determined.

MATERIALS AND METHODS

Materials

Soybeans, *Glycine max* (L.) Merrill, were provided by Frutarom (Londerzeel, Belgium). Acetic acid (HOAc) (HPLC grade), daidzein, genistein, coumestrol, L-leucine, L-histidine, sodium hydroxide (NaOH) pellets and 17β-estradiol (E_2) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol (100%, analytical grade), D-(+)-glucose (dextrose), ammonium sulphate and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany) and yeast nitrogen base without amino acids & without ammonium sulphate, and agar were obtained from Becton-Dickinson (Franklin Lakes, NJ, USA). HOAc (ULC/MS grade), acetonitrile (ACN) (ULC/MS and HPLC-R grade), silica gel (60Å, 70-230 mesh), water (ULC/MS grade), ethyl acetate (LC-MS grade), and methanol (ULC/MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water for other purposes than UHPLC was

prepared using a Milli-Q water purification system (Millipore, Molsheim, France). The reporter yeast strain was provided by RIKILT (Wageningen, The Netherlands).

Rhizopus-challenged germination and extraction of soybean seedlings

Soybeans, *Glycine max* (L.) Merril, were germinated and challenged with the fungus *Rhizopus microsporus var. oryzae* (LU581) as described previously.[2] After germination and challenging, the soybean sprouts were freeze-dried and milled with a Retsch Ultra centrifugal mill ZM (Haan, Germany), using a 0.5 mm sieve. Soybeans were defatted by hexane. Next, isoflavonoids were extracted with ethanol and subsequent drying was performed as described elsewhere.[2] The dried extract containing the isoflavonoids is referred to as soy extract.

Heat treatment of soybean extract

The soybean extract was suspended in either 0.56 M HOAc or water to a concentration of 10.7 mg/mL. The suspension was heated in a water bath of 100 °C, during three independent experiments. Samples were taken after 20, 30, 40, 50, 60, and 70 min in experiment 1; after 10, 30, 60, and 120 min in experiment 2; after 1, 5, 30, 60, 180, and 240 min in experiment 3. After the heat treatment, ethanol (EtOH) was added to obtain a 70% (v/v) EtOH concentration. Solubilisation was enhanced in a water bath of 40 °C for 5 min and 10 min sonication at 25 °C. Finally, the samples were centrifuged (18.500 x g; 5 min; RT) and the supernatant was stored at -20 °C.

RP Flash chromatography

For Flash chromatography, the untreated soybean extract and a modified soybean extract (0.56 M HOAc, 70 min at 100 °C) were used. Solvents were evaporated under vacuum, frozen and lyophilized. A Grace Reveleris™ Flash system was used to obtain fractions enriched in glyceollins or dehydroglyceollins. The untreated and treated soybean extract (52.2 mg DW) were solubilised in methanol acidified with 0.1% (v/v) HOAc to a concentration of 5 mg/mL (5 min in a water bath of 40 °C, followed by 10 min sonication at 25 °C). This solution was added to 3 g silica and the soybean extract was coated onto silica particles by using a rotary evaporator. The soybean extract-coated silica was transferred into an empty 5 g cartridge and closed with a plunger. The cartridge was placed upstream of a 12 g Reveleris C18 RP column (particle size 38.6 µm) (Grace Davison Discovery Science, Columbia, MD, USA). Water (Milli-Q) acidified with 0.1% (v/v) HOAc (HPLC grade) + 1% (v/v) ACN (HPLC-grade), eluent A, and ACN (HPLC-grade) acidified with 0.1% (v/v) HOAc (HPLC grade), eluent B, were used as eluents. The flow rate was 30 mL/min and the experiment was performed at

room temperature. The following elution profile was used: 0-5 min, linear gradient from 0-40% B; 5-9 min, linear gradient from 40-50% B; 9-14 min, isocratic on 50% B; 14-19 min, linear gradient from 50-60% B; 19-24 min, linear gradient from 60-70% B; 24-27 min, linear gradient from 70-80% B; 27-28 min, linear gradient from 80-84% B; 28-33 min, linear gradient from 84-96% B; 33-35 min, linear gradient from 96-100% B. Absorption was measured at 280 nm. During the entire run, fractions of 10 mL were collected. All fractions were analyzed with UHPLC-MS. Fractions containing similar glyceollins or dehydroglyceollins were pooled. Solvents from pools containing dehydroglyceollins were evaporated under vacuum, frozen and lyophilized. Ethyl acetate was added to the pools containing glyceollins in a ratio 3:1 (v/v). This liquid-liquid partitioning was performed three times. The pooled ethyl acetate fractions were evaporated under vacuum, frozen and lyophilized. The pools were re-solubilised in 70% (v/v) ethanol for analysis with UHPLC-MS and re-solubilised in DMSO to determine the estrogenicity.

RP-UHPLC-ESI-MS analysis

Samples were analyzed on an Accela ultra high performance liquid chromatography (RP-UHPLC) system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, autosampler and PDA detector. Samples (5 µL for the soybean extract and 1 µL for isoflavonoid-enriched pools) were injected on an Acquity UPLC BEH Shield RP18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity UPLC Shield RP18 Vanguard guard-column (2.1 x 5 mm, 1.7 µm particle size; Waters, Milford, MA, USA). Water acidified with 0.1% (v/v) HOAc + 1% (v/v) ACN, eluent A, and ACN acidified with 0.1% (v/v) HOAc, eluent B, were used as eluents. The flow rate was 300 µL/min, the column temperature was controlled at 35 °C, and the PDA detector was set to measure 200-400 nm. The following elution profile was used: 0-1 min, isocratic on 9% B; 1-3 min, linear gradient from 9-25% B; 3-10 min, linear gradient from 25-50% B; 10-13 min, isocratic on 50% B; 13-23 min, linear gradient from 50-100% B; 23-25 min, isocratic on 100% B; 25-26 min, linear gradient from 100-9% B; 26-31 min, isocratic on 9% B.

Mass spectrometric (MS) data were obtained by analyzing samples on a LTQ-Velos (Thermo Scientific) equipped with a heated ESI-MS probe coupled to the RP-UHPLC. Nitrogen gas was used as sheath and auxiliary gas. Data were collected over a *m/z* range of 150-1500 in both positive (PI) and negative (NI) mode. Data-dependent MS^2 analysis was performed with a normalised collision energy of 35%. Most settings were optimized via automatic tuning using "Tune Plus" (Xcaliber 2.1, Thermo Scientific). The system was tuned with genistein in both PI and NI mode. For the PI an NI mode, the source voltage were 4.50 kV and 3.50 kV, respectively. In both ionisation

modes the ion transfer tube (ITT) temperature and the probe temperature were 400 °C and 50 °C, respectively.

The identification of isoflavonoids was based on UV and MS spectra using the approach reported earlier.[1,2,12] The quantification of isoflavonoids was performed based on their absorption at 280 nm by means of Xcalibur (version 2.1.0, Thermo Scientific). As for many compounds no commercial standards were available, the amounts of isoflavonoid were expressed as mg daidzein equivalents per gram challenged soybean seedling extract, in which daidzein was used as a generic standard to make a calibration curve with five data points (0.001-0.01 mg/mL, $R^2 = 0.996$).

Determination of molecular characteristics

Molecular structures were evaluated using the software program ChemBio3D Ultra 12 (Cambridgesoft, Waltham, MA, USA) after MM2 molecule minimization.

Determination of estrogenic activity

Estrogenicity was determined as described elsewhere[13] with slight modifications. Cultures of the yeast estrogen biosensor with either hERα or hERβ were grown overnight at 30 °C and 200 rpm. At the late log phase, both cultures were diluted in minimal medium supplemented with either L-leucine (hERα) or L-histidine (hERβ) to an optical density value at 630 nm (OD_{630}) of 0.045±0.005 and 0.200±0.005 for hERα and hERβ, respectively. For determination of ER agonism, 200 µL aliquots of diluted yeast cultures were combined with 2 µL of test compound or isoflavonoid fraction in a 96-well plate. Dilution series of each sample were prepared in DMSO ranging from 0.0001–10 µg/mL. The final concentration of DMSO in the assay did not exceed 1% (v/v). Each sample concentration was assayed in triplicate. Exposure was performed for 6 or 24 h for the hERβ or hERα assay, respectively, at 30 °C and orbital shaking at 200 rpm. Fluorescence and OD were measured at 0 and 24 h for the hERα and at 0 and 6 h for the hERβ in a Tecan Infinite F500 (Männedorf, Switzerland), using an excitation filter of 485 nm (bandwidth, 20 nm) and an emission filter of 535 nm (bandwidth, 35 nm). The fluorescence signals of the samples were corrected with the signal obtained with the diluted yeast suspension at t=0. In order to verify the viability of the yeast in each well, the absorbance was measured at 630 nm. Cytotoxicity occurred when the yeast growth was inhibited for more than 30% during the incubation and these incubations were not further considered. DMSO (blank) and control samples containing 17β-estradiol (E_2) dissolved in DMSO were included in each experiment. The EC_{50} values for 17β-estradiol in the hERα and hERβ bioassay were 0.66 nM and 0.18 nM, respectively, and were in line with those reported previously.[13] EC_{50} calculations were performed in Sigma Plot (8.02, SPSS Inc., Chicago,

IL, USA). The relative estrogen potency (REP) of a compound was determined by dividing the EC_{50} of E2 by the EC_{50} of the compound. The EC_{50} values could not be calculated in all cases, due to toxicity of the compounds towards the yeast. To be consistent for all compounds, the REP was determined by thresholds, *i.e.* the threshold of E_2 divided by the threshold of the compound. The threshold value was defined as the value where the slope was > 0.4 units relative absorbance per log difference in concentration. The threshold values for 17β-estradiol in the hERα and hERβ bioassay were 5.44×10^{-8} mg/mL and 2.45×10^{-8} mg/mL, respectively. For the determination of ER antagonism, the yeast cells were exposed to 2 µL of EC_{70} (hERα) or EC_{90} (hERβ) of 17β-estradiol in combination with 2 µL of different dilutions of test compound or fraction (measured in triplicates). For antagonism the decrease of the maximal response in percentages was determined.

RESULTS

Identification of isoflavonoids in untreated and heated soybean extracts

Comparison of the chromatograms of the 60 min acid/heat treated and untreated soybean extract showed that a number of compounds were acid/heat unstable (Figure 2.2). In total, 42 peaks were distinguished using UV, MS and MS^2 in negative and positive mode (Table 2.1), many of which were annotated in our previous studies.[1,2] Peaks 22, 30 and 34-38 and 40 were new. They seemed to be formed upon acid/heat treatment at the expense of peaks 3, 8, 17, 19-21, 25 and 29, which all represented 6a-hydroxy-pterocarpans. The newly formed compounds had molecular masses minus 18 Da compared to the original 6a-hydroxy-pterocarpans. Moreover, the newly formed compounds showed a second absorption maximum between 330 and 364 nm, besides the one at around 280 nm (Table 2.1). This indicated that these newly formed compounds have a larger conjugated system than the 6a-hydroxy-pterocarpans. Taken together, this might suggest that the 6a-hydroxy-pterocarpans were converted into 6a,11a-pterocarpenes. Formation of 6a,6-pterocarpenes seems unlikely as these compounds have a less extensive conjugated system, consistent with their lower λmax of 302 nm.[14] For 6a,11a-pterocarpenes, other than those from soybean, absorption maxima between 330 and 350 nm have been reported, similar to those observed in the present study.[15]

MS and MS^2 NI mode. Compound **22** with an *m/z* value of 253 in NI mode produced fragment ions *m/z* 209 $[M - H - CO_2]^-$, 225 $[M - H - CO]^-$, and 235 $[M - H - H_2O]^-$. It was tentatively assigned as dehydroglycinol. Compound **30** was tentatively assigned as dehydroglyceofuran based on a *m/z* of 335 in NI mode and fragment ions with a *m/z* of 291 $[M - H - CO_2]^-$, and 307 $[M - H - CO]^-$. Compound **34** was tentatively

identified as dehydroglyceollidin II with a m/z of 321 in NI mode. In NI MS^2 mode fragment ions *m/z* 265 $[M - H - 2CO]^-$, 277 $[M - H - CO_2]^-$, and 306 $[M - H - CH_3]^-$ were formed. Compounds **35-37** were tentatively assigned as dehydroglyceollin III, II and I, as these compounds have a m/z of 319 in NI mode. They produced the fragment ions *m/z* 291 $[M - H - CO]^-$ and 275 $[M - H - CO_2]^-$ in NI MS^2 mode. Assuming that compounds **35-37** had the same elution order as their corresponding glyceollins, peak **35** was tentatively annotated as dehydroglyceollin III, peak **36** as dehydroglyceollin II, and peak **37** as dehydroglyceollin I. Compound **38** was tentatively annotated as dehydroglyceollin IV with a *m/z* of 335 in NI mode. In NI MS^2 a loss of a methyl radical was observed. This is indicative for the presence of a methoxy group.[16] Compound **40** had a *m/z* of 317 in NI mode and was tentatively identified as dehydroglyceollin VI, with neutral losses of CO (28 Da) and CO_2 (44 Da). In this study, only traces of *retro*-Diels Alder fragments were observed in NI MS^n for the compounds described above. Such MS behaviour was also found in other studies performed with pterocarpenes and coumestans.[15,17] Both these subclasses of isoflavonoids have a relatively large conjugated system. This results in small neutral losses and no apparent A-ring or B-ring fragment ions, due to the resistance against cleavage of the C-ring or D-ring.

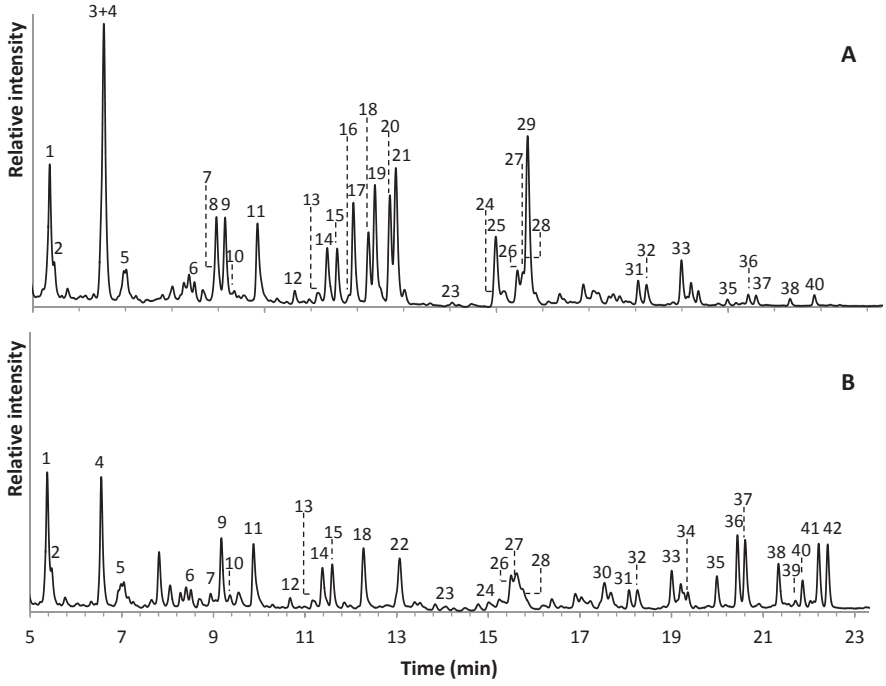

Figure 2.2. RP-UHPLC-UV profile (280 nm) of untreated (A) and heat (60 min)/acid treated (B) soybean extract. Peak numbers refer to compounds in Table 2.1.

MS and MS^2 PI mode. Additional data for confirmation that the prenylated compounds after the treatment were formed at the expense of the 6a-hydroxy-pterocarpans came from PI MS analysis, which allowed prediction of the type of prenylation.[18] For compounds **34**-**38** and **40** neutral losses of 42 Da and 56 Da were observed in PI MS^2. The ratio (42:56) was <1 for compound **34** and **38,** which suggested that these compounds had a prenyl chain. This ratio was >1 for compounds **35** and **36,** indicative for a pyran ring. Compounds with a furan ring are known to show a different behaviour.[17] Glyceollin III has a 2''-isopropenyl-dihydrofuran-group and the ratio (42:56) is >1. On the other hand, glyceollin VI has a 2"-isopropenyl-furano-group and the ratio is <1. Upon fragmentation of compounds **35** and **40**, which were probably formed from glyceollin III and glyceollin VI, respectively, it was observed that the ratio for compound **35** was >1 and that for compound **40** was <1. This is in line with the corresponding 6a-hydroxy-pterocarpans. Based on UV data, MS^n data in negative and positive mode the compounds were tentatively annotated as 6a,11a-pterocarpenes. It seems that the 6a-hydroxyl group of the original 6a-hydroxy-pterocarpans was first protonated, followed by dehydration and formation of a double bond during the heat treatment. This resulted in a more extensive conjugated system than the original 6a-hydroxy-pterocarpan. These findings are in accordance with previous data.[11] They suggest that the 6a-hydroxy-pterocarpan glyceollin I forms the thermodynamically more stable 6a,11a-pterocarpene dehydroglyceollin I upon water loss.[11] Structures of the main prenylated 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes are shown in Figure 2.1. Note that the nomenclature of glyceollins and dehydroglyceollins is ambiguous in literature, as elaborated in the footnote of Table 2.1.

Peaks **39**, **41** and **42** appeared only after 20 min of acid/heat treatment. They all had a *m/z* value in NI mode of 277.2169, corresponding to the molecular formula of $C_{18}H_{30}O_2$. Similar *m/z* values suggest that compounds **39**, **41** and **42** were isomers. Despite the fact that the amount of these compounds increases and the amount of dehydroglyceollin I, II and III decreases after longer duration of incubation, it is unlikely that there is a relationship between these compound groups, considering the molecular formulas of dehydroglyceollins ($C_{20}H_{16}O_4$) and compounds **39**, **41** and **42**.

Table 2.1. Compounds tentatively assigned in the soybean extracts by UHPLC-ESI-MS[a].

No[b]	Rt. (min)	UV_{max} (nm)	Identification	Subclass	Molecular formula	$[M-H]^-$	MS^2 product ions (relative intensity)[c]	$[M+H]^+$	MS^2 product ions (relative intensity)[b]
1	5.56	250	Daidzin	Isoflavone	$C_{21}H_{20}O_9$	415	253(100)	417	255(100)
2	5.66	257	Glycitin	Isoflavone	$C_{22}H_{22}O_{10}$	445	283(100)	447	285(100)
3	6.73	283	Glycinol	Pterocarpan	$C_{15}H_{12}O_5$	271	161(100), 227(25)	255[c]	199(100), 227(66), 237(11)
4	6.74	260	Genistin	Isoflavone	$C_{21}H_{20}O_{10}$	431	296(100), 311(8)	433	271(100)
5	7.22	257	Biochanin A	Isoflavone	$C_{16}H_{12}O_5$	283	268(100)	285	270(100)
6	8.69	260	Acetyl-genistin	Isoflavone	$C_{27}H_{30}O_{15}$	473	269(100)	475	271(100)
7	9.14	256	Glycitein	Isoflavone	$C_{16}H_{12}O_5$	283	268(100)	285	257(14), 270(100)
8	9.16	257, 291	Glyceofuran	Pterocarpan	$C_{20}H_{18}O_6$	353	335(100), 149(13)	337[c]	188(39), 309(100), 319(76)
9	9.35	248	Daidzein	Isoflavone	$C_{15}H_{10}O_4$	253	209(58), 225(100), 253(45)	255	199(100), 227(61), 237(25)
10	9.55	286	2′-hydroxygenistein	Isoflavone	$C_{15}H_{10}O_6$	285	199(10), 217(100), 241(10)	287	217(100), 259(49), 269(20)
11	10.05	286, 353	C-methyl-coumestrol	Coumestan	$C_{16}H_{10}O_5$	281	253(100)	283	255(100)
12	10.85	286	Naringenin	Flavanone	$C_{15}H_{12}O_5$	271	151(100), 177(22)	273	153(100)
13	11.35	257	Prunetin	Isoflavone	$C_{16}H_{12}O_5$	283	240(24), 255(100), 268(11)	285	229(7), 257(100), 267(2)
14	11.55	350	Isotrifoliol	Coumestan	$C_{16}H_{10}O_6$	297	282(100)	299	267(8), 271(100), 284(20)
15	11.77	260	Genistein	Isoflavone	$C_{15}H_{10}O_5$	269	201(63), 225(100), 241(34)	271	153(100), 215(74), 243(65), 253(35)
16	12.01	285	Glyceollidin I	Pterocarpan	$C_{20}H_{20}O_5$	339	161(100), 324(54)	323[d]	267(100)
17	12.12	285	Glyceollidin II	Pterocarpan	$C_{20}H_{20}O_5$	339	161(100), 324(45)	323[d]	267(100)
18	12.44	304, 343	Coumestrol	Coumestan	$C_{15}H_8O_5$	267	211(11), 239(100), 267(24)	269	197(26), 225(30), 241(100)
19	12.58	289	Glyceollin III	Pterocarpan	$C_{20}H_{18}O_5$	337	149(14), 293(6), 319(100)	321[d]	251(98), 279(100), 306(73)
20	12.90	283	Glyceollin II	Pterocarpan	$C_{20}H_{18}O_5$	337	149(37), 293(22), 319(100)	321[d]	251(81), 279(100), 306(79)
21	13.03	283	Glyceollin I	Pterocarpan	$C_{20}H_{18}O_5$	337	149(78), 293(34), 319(100)	321[d]	293(38), 303(100), 306(84)
22	13.26	284, 352, 357	Dehydroglycinol	Pterocarpene	$C_{15}H_{10}O_4$	253	209(61), 225(100), 235(36)	255	199(30), 213(22), 227(100)
23	14.25	288	A-ring prenylated 2′-hydroxydaidzein	Isoflavone	$C_{20}H_{20}O_5$	337	282(88), 293(100), 337(37)	339	283(100)
24	15.17	257	B-ring prenylated glycitein	Isoflavone	$C_{21}H_{20}O_5$	351	336(100)	353	285(9), 297(100)
25	15.19	287, 308	Glyceollin VI	Pterocarpan	$C_{20}H_{16}O_5$	335	149(16), 317(100)	319[d]	263(100), 291(72), 319(11)

26	15.65	252	A-ring prenylated daidzein	Isoflavone	$C_{20}H_{18}O_4$	321	266(100)	323	267(100)
27	15.77	262	A-ring prenylated 2'-hydroxygenistein	Isoflavone	$C_{20}H_{18}O_6$	353	267(38), 284(93), 285(100)	355	299(100)
28	15.84	257	B-ring prenylated daidzein	Isoflavone	$C_{20}H_{18}O_4$	321	265(100), 252(4)	323	255(11), 267(100)
29	15.87	285	Glyceollin IV	Pterocarpan	$C_{21}H_{22}O_5$	353	148(5), 149(14), 335(100)	337[c]	281(100), 269(50)
30	17.78	273, 331	Dehydroglyceofuran	Pterocarpene	$C_{20}H_{16}O_5$	335	280(100), 291(14), 307(30)	337	281(100), 309(8)
31	18.26	264	A-ring prenylated genistein	Isoflavone	$C_{20}H_{18}O_5$	337	282(100), 283(8), 309(4)	339	283(100)
32	18.44	261	B-ring prenylated genistein	Isoflavone	$C_{20}H_{18}O_5$	337	281(100), 282(19), 337(65), 293(13)	339	271(18), 283(100)
33	19.19	307, 343	4-prenyl-coumestrol	Coumestan	$C_{20}H_{16}O_5$	335	280(100), 281(12)	337	281(100)
34	19.46	286, 340, 355	Dehydroglyceollidin II	Pterocarpene	$C_{20}H_{18}O_4$	321	265(100), 277(12), 306(12)	323	267(100), 281(20)
35	20.19	296, 344, 359	Dehydroglyceollin III	Pterocarpene	$C_{20}H_{16}O_4$	319	275(24), 277(100), 291(22)	321	253(53), 279(48), 293(75), 306(100)
36	20.64	273, 349, 364	Dehydroglyceollin II	Pterocarpene	$C_{20}H_{16}O_4$	319	275(38), 277(100), 291(90), 304(85)	321	253(19), 279(33), 293(100), 306(52)
37	20.81	288, 332	Dehydroglyceollin I	Pterocarpene	$C_{20}H_{16}O_4$	319	275(32), 291(100), 304(38)	321	279(28), 293(100), 306(74)
38	21.54	292, 340, 356	Dehydroglyceollin IV	Pterocarpene	$C_{21}H_{20}O_4$	335	320(100)	337	281(30), 295(15), 321(100)
39	21.91	273, 290	Unknown degradation product	-	$C_{18}H_{30}O_2$	277	179(10), 233(100), 259(38)	279	123(69), 137(48), 223(50), 261(100)
40	22.06	280, 355	Dehydroglyceollin VI	Pterocarpene	$C_{20}H_{14}O_4$	317	273(20), 289(100)	319	275(50), 291(52), 304(100)
41	22.41	262, 271, 282	Unknown degradation product	-	$C_{18}H_{30}O_2$	277	179(9), 233 (100), 259(28)	279	123(39), 137(41), 223(42), 261(100)
42	22.61	259, 268, 279	Unknown degradation product	-	$C_{18}H_{30}O_2$	277	179(10), 233 (100), 259(24)	279	123(40), 137(40), 223(42), 261(100)

[a] The nomenclature of glyceollins and dehydroglyceollins is ambiguous. The nomenclature in this article is based on Simons *et al.* and Aisyah *et al.*[1,2] Glyceollin VI in this article is sometimes also referred to as clandestacarpin in the literature. Dehydroglyceollin III in this article is also referred to as glyceollin VI in the literature. In the current work, 'glyceollin' always refers to prenylated 6a-hydroxy-pterocarpans, whereas 'dehydroglyceollin' always refers to prenylated 6a,11a-pterocarpenes. [b] Numbers refer to peaks in Figure 1.2. [c] Only peaks with a relative intensity of 4% or higher were considered, with a maximum of the four most abundant product ions. [d] The $[M+H-H_2O]^+$ dominated in the MS^1 compared to the $[M+H]^+$.

Stability of 6a-hydroxy-pterocarpans compared to other soybean isoflavonoids

The soybean extract contained different subclasses of isoflavonoids (Table 2.1). Before heating, 6a-hydroxy-pterocarpans comprised 45% of the total area at 280 nm of all isoflavonoids, coumestans 10%, isoflavones 43%, and pterocarpenes 1%. After a 60 min combined heat/acid treatment all 6a-hydroxy-pterocarpans were converted. Now, 29% of the total area of isoflavonoids belonged to the subclass of pterocarpenes, 20% corresponded to coumestans, and 51% to isoflavones. The compositions were expressed as percentages of the total peak area, as molecular extinction coefficients were not known for all compounds. Comparing the total peak area of the untreated soybean extract and the treated soybean extract (60 min 100 °C + 0.56 M HOAc), it can be calculated that a loss of 45% of the total isoflavonoids occurred during acid/heat treatment (Figure 2.2). When only heat was applied a loss of 19% was observed (data not shown). Considering the scope of this study, this loss of isoflavonoids was not further elaborated. The conversion of isoflavonoids when heated with and without acid at different time points can be seen in Figure 2.3.

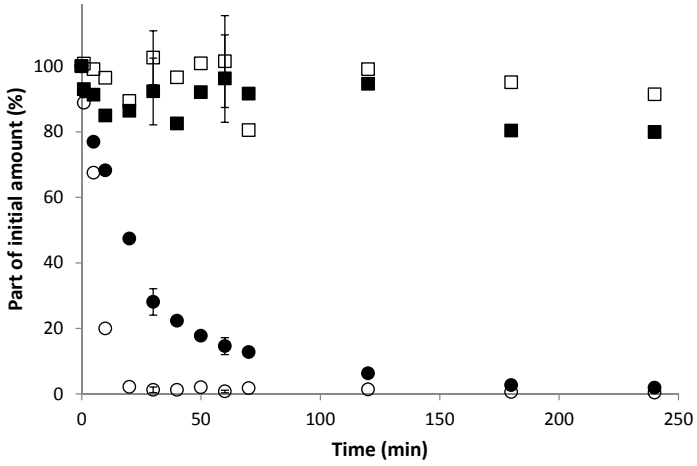

Figure 2.3. Conversion of 6a-hydroxy-pterocarpans in time with (o) or without acid (●) and the conversion of other isoflavonoids in time with (□) or (■) without acid.

The 6a-hydroxy-pterocarpans were converted for 23% after 5 min, and for 53% after 20 min; within 180 min at 100 °C all 6a-hydroxy-pterocarpans were converted. This transition was accelerated with acid: after 5 min 32% was converted and within 20 min all 6a-hydroxy-pterocarpans were converted. This is in agreement with a previous study, showing that glyceollin I was unstable at temperatures above 40 °C and at pH values below 4.[11] The other three subclasses of isoflavonoids showed little

conversion (~15%) and acid did not accelerate this conversion. This suggests that the 6a-hydroxyl group of 6a-hydroxy-pterocarpans in particular is susceptible to acid. This is in line with a thermal degradation study, in which daidzein and genistein did not degrade after 3 h of heating at 150 °C, whereas a minimal degradation of glycitein was seen after 1 h of heating.[19] In contrast, it has also been shown[20] that degradation of daidzein and genistein, occurred at 120 °C and pH 7 and 9.

Preparative enrichment of prenylated 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes

In order to compare the estrogenic properties of glyceollins and dehydroglyceollins, the (treated) soybean extracts were fractionated using Flash chromatography, with a fairly good separation considering the particle size of the column material (Figure 2.4).

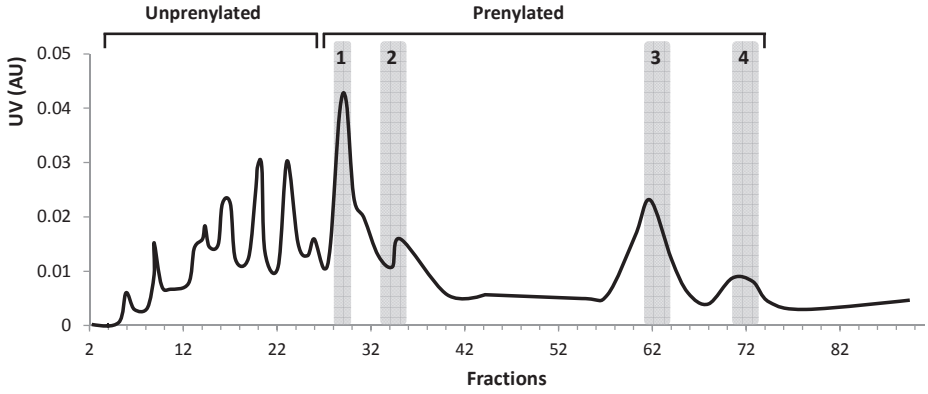

Figure 2.4. Flash chromatography UV profile (280 nm) of the mildly treated soybean extract. Grey areas refer to the four different pools collected.

All fractions were analyzed by RP-UHPLC-MS and it was seen that the first 20 fractions contained unprenylated isoflavonoids (data not shown). The subsequent fractions contained prenylated isoflavonoids and the compositional analysis of these fractions is shown in Figure 2.5. Different prenylated isoflavonoids could be separated and different pools were made based on this compositional analysis (Figure 2.4). Fractions 28-29 and 33-36 contained the prenylated 6a-hydroxy-pterocarpans, mainly present before the treatment. Fractions 28 and 29 were combined to obtain pool 1, and 97% of this pool was comprised of the 6a-hydroxy-pterocarpans glyceollin I, II and III. Pool 2 (fractions 33-36) contained 74% glyceollin IV and VI.

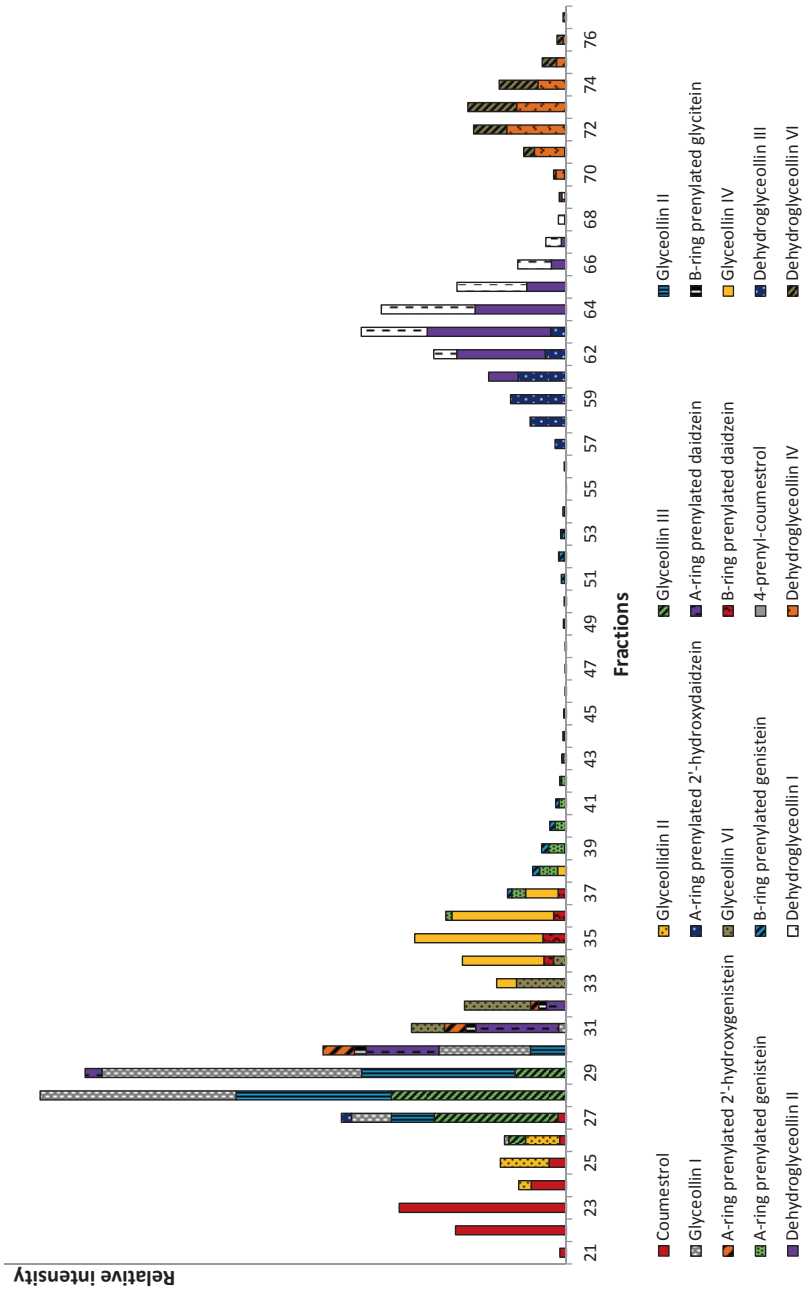

Figure 2.5. Content of Flash chromatography fractions of the mildly treated soybean extract determined by RP-UHPLC-MS.

Fractions 58-66 and 71-75 contained the prenylated 6a,11a-pterocarpenes, obtained in high quantities after the heat/acid treatment. Fractions 58-66 were combined to obtain pool 3, which consisted of 88% dehydroglyceollin I, II and III. Pool 4 (fractions 71-75) contained 89% dehydroglyceollin IV and VI (Table 2.2 and Figure S2.1). It should be noted that the sample used for Flash chromatography was dried at slightly elevated temperatures, which resulted in a larger proportion of 6a,11a-pterocarpenes than in the untreated sample shown in Figure 2.2A. This sample is further referred to as 'mildly treated'. The treated sample used for Flash chromatography had a further increased proportion of 6a,11a-pterocarpenes (pool 3 and 4) compared to the untreated sample, at the expense of 6a-hydroxy-pterocarpans (pool 1 and 2) (data not shown).

Table 2.2 Abundance of prenylated isoflavonoids in the enriched pools.

Pool	Identification	Area 280 nm (%)
1	Glyceollin III	15.3
	Glyceollin II	31.1
	Glyceollin I	51.0
	Other	2.6
2	Glyceollin VI	0.9
	Glyceollin IV	72.6
	Other	26.5
3	Dehydroglyceollin III	10.3
	Dehydroglyceollin II	38.1
	Dehydroglyceollin I	39.3
	Other	12.3
4	Dehydroglyceollin IV	81.2
	Dehydroglyceollin VI	7.9
	Other	10.9

Change in estrogenic activities upon heating

The different pools were tested for their estrogenic activities (Figure 2.6). Agonistic activities were expressed as relative estrogen potencies (REPs) based on the threshold values, and antagonistic activities were expressed as the decrease of the maximal response in percentages. Pool 1 (glyceollin I-III) showed a slight ER agonistic activity towards hERα (REP 1.1 x 10^{-5}) and a clear agonistic activity towards hERβ (REP 1.2 x 10^{-4}). No antagonistic properties of pool 1 could be established towards both receptors (Figures 2.6A and 2.6C). Estrogenic activity of glyceollin I-III seemed ambiguous, as it was previously shown that glyceollin I, II and III could act as agonist and antagonist towards estrogen receptors.[8-10] Taken together, the present results and those from previous studies[8-10] indicated that glyceollin I, II and III might act as SERMs. Pool 2, containing glyceollin IV and VI, showed a slight agonistic activity

towards hERα (REP 1.1 x 10^{-5}), a clear agonistic activity towards hERβ (REP 1.0 x 10^{-4}), and slight antagonistic activities towards hERα and hERβ (34% and 24%, respectively) (Figures 2.6A and 2.6C), indicating that glyceollin IV and VI are partial agonists or can act as SERMs.[21] Other assays, *e.g.* proliferation assays based on human breast cells or U2-OS based hER-CALUX bioassays, are needed to conclude on the exact estrogenic classification.

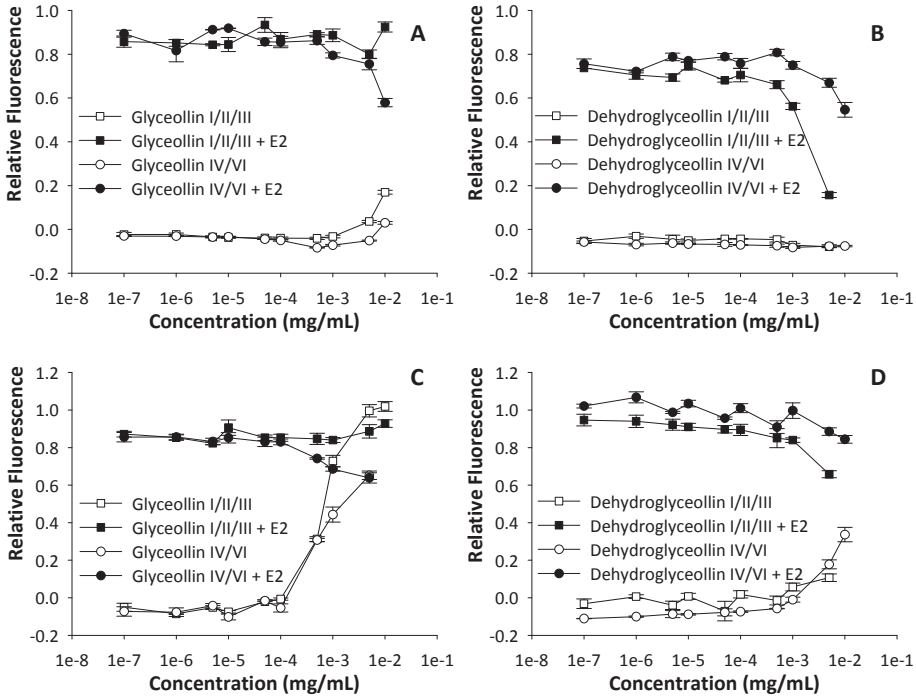

Figure 2.6. Transcriptional activation by hERα (A and B) and hERβ (C and D) in response to pools 1-4.

Figures 2.6B and 2.6D show the estrogenic activities of the corresponding dehydroglyceollins (pools 3 and 4). Figure 2.6B shows that the dehydroglyceollins had clear antagonistic properties towards hERα, *i.e.* 78% decrease with pool 3 (dehydroglyceollin I, II and III) and 26% decrease with pool 4 (dehydroglyceollin IV and VI), whereas no hERα agonistic activity was observed (Figure 2.6B). Towards hERβ, dehydroglyceollins seemed to have both agonistic and antagonistic properties (Figure 2.6D), indicating that these compounds could act as partial agonists or as SERMs. No agonistic response was detected for dehydroglyceollin I-III, whereas the REP value of dehydroglyceollin IV/VI was 9.8 x 10^{-5}. The antagonistic effects resulted in a 28% decrease in estrogenic activity for dehydroglyceollin I-III and 15% for dehydroglyceollin IV/VI. In summary, glyceollins showed higher agonistic activity

towards hERβ than towards hERα, which is in line with literature[4]. In particular, the response towards hERα changed from agonistic for glyceollin I-III to full antagonistic for dehydroglyceollins I-III. Towards hERβ a decrease in agonistic activity was observed after heating.

DISCUSSION

Compounds are considered estrogenic when they activate the hER at concentrations below 10^4 times that of estradiol.[22] Thus, REP values ≥ $1x\ 10^{-4}$ indicate that compounds (or pools) have clear estrogenic properties, which was not always the case with the pools tested in the present study. Intriguingly, the estrogenic properties of glyceollins and their corresponding dehydroglyceollins were different.

After heat/acid treatment, the dehydroglyceollins that were formed from 6a-hydroxy-pterocarpans showed full antagonistic properties towards hERα and lower agonistic activities towards hERβ. It has been suggested[23] that the removal of the 6a-hydroxyl group with the concomitant extra planarity of the dehydroglyceollins might increase the binding to the estrogen receptor. This was not observed in the present study. Nevertheless, our results clearly showed that dehydroglyceollins had different mode of action towards both receptor types compared to the 6a-hydroxy-pterocarpans. The natural ligand 17β-estradiol has two hydroxyl groups, each on one side of the molecule. These are required for binding to the estrogen receptor, the distance of which is important for binding. When the geometry of glyceollins and dehydroglyceollins is compared (Figure 2.7), it is apparent that the length of the two compounds is different.

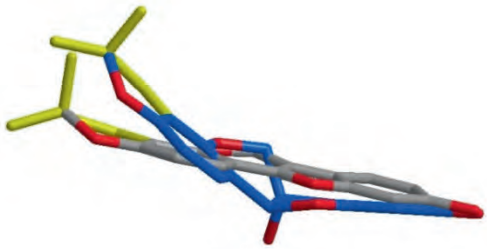

Figure 2.7. Molecular structures of glyceollin I (blue) and dehydroglyceollin I (grey). Prenyl group is indicated in yellow.

With the increased planarity of the dehydroglyceollins, the distance between the two hydroxyl groups of dehydroglyceollins becomes larger than that in glyceollins. Furthermore, the orientation of the prenyl group changes between the two compounds as a result of the planarity. This might affect binding in the ligand-binding domain of the receptor, turning the agonistic response observed for glyceollins into

the antagonistic response observed for dehydroglyceollins in case of hERα, and into a decreased agonistic response observed for dehydroglyceollins in case of hERβ.

Glyceollins showed agonistic activities to both estrogen receptors. This gives the opportunity to use these glyceollins of elicited soybean sprouts in food supplements to prevent menopausal complaints. It is known that dietary agonistic estrogens, like soybean isoflavones, are helpful to counteract the drop in endogenous estrogen levels in postmenopausal women.[6,24] The present results show that care should be taken during processing (*e.g.* when soybean glyceollins are processed into food supplements) or isolation, because 6a-hydroxy-pterocarpans are prone to transformation upon which estrogenic characteristics can change, *i.e.* drop in estrogenic activity or switch from agonistic to antagonistic behaviour.

Alternatively, heat and acid might be used to modulate estrogenicity of elicited soy sprouts in a relatively simple way. After the treatment, the dehydroglyceollins showed full antagonistic activity towards hERα. Full antagonistic compounds, like fulvestrant and RU 58668, are well-known molecules in treatment of patients with breast cancer.[21,25,26] Full antagonists are rare, and dehydroglyceollins might represent a new class of hERα antagonists, although further verification of their *in vitro* anti-estrogenic potency by other bioassays and their *in vivo* activity remain to be established.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

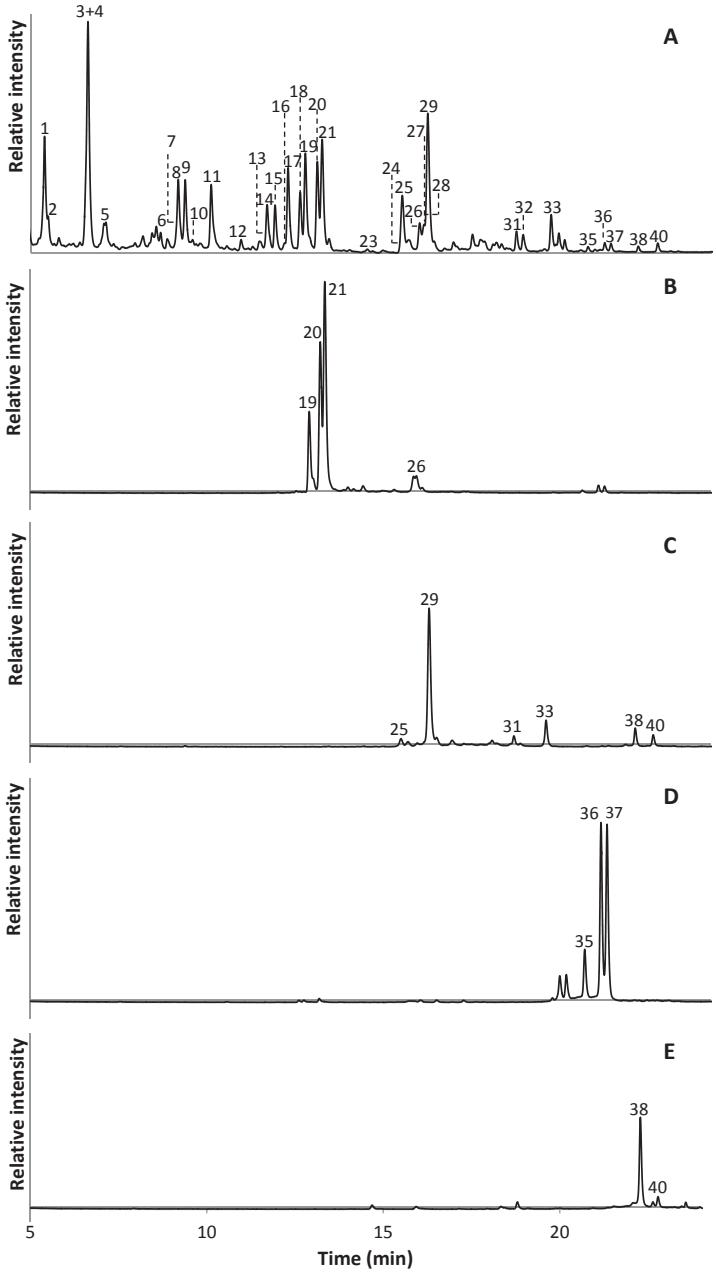

Figure S2.1. RP-UHPLC-UV profile (280 nm) of the untreated soy extract (A), pool 1 (B), pool 2 (C), pool 3 (D), and pool 4 (E). Peak numbers refer to compounds in Table 2.1.

Chapter 3

Involvement of a hydrophobic pocket and helix 11 in determining the mode of action of prenylated flavonoids and isoflavonoids in the human estrogen receptor

ABSTRACT

Six prenylated (iso)flavonoids were purified from a licorice root extract and were completed with six commercially available (iso)flavonoids. The agonistic and antagonistic activities of these compounds towards both hERα and hERβ were determined. Differences in the modes of action (agonist or antagonist) were observed for the various compounds tested. In general, each compound had the same mode of action towards both ERs. *In silico* modelling was performed in order to study the differences in estrogenicity observed between the compounds. This suggested that prenyl chains fit into a hydrophobic pocket present in the hER, which results in an increased agonistic activity. Besides, it was shown that an increase in length (~1.7 Å) of pyran prenylated isoflavonoids resulted in an antagonistic mode of action. This might be caused by collision of the pyran ring with helix 11 in the ligand binding cavity of the hER.

Based on: Milou G.M. van de Schans, Tina Ritschel, Toine F.H. Bovee, Mark G. Sanders, Pieter de Waard, Harry Gruppen, Jean-Paul Vincken. ***Submitted for publication*.**

INTRODUCTION

Phenyl benzopyrans are plant-derived molecules, which are characterized by a C_6-C_3-C_6 carbon structure. Flavonoids (2-phenyl benzopyrans) and isoflavonoids (3-phenyl benzopyrans) differ in the position of their B-ring. They comprise various subclasses, amongst others flavones, flavanones and flavonols (all flavonoids), isoflavones, isoflavans and isoflavenes (all isoflavonoids), which differ in the configuration of the C-ring.[3] Flavonoids and isoflavonoids can be prenylated at different positions with different kinds of prenyl groups, *i.e.* chain, furan and pyran. Increased intake of flavonoids and isoflavonoids is often correlated with decreased risk of diseases related to a Western lifestyle.[4-7] It has been demonstrated that flavonoids and isoflavonoids can interact with the human estrogen receptors (hERs), which is often considered a critical step in their presumed beneficial effects.[9,10]

Binding of ligands to hERs can result in different modes of action: agonistic (full or partial), antagonistic (direct or indirect) and selective estrogen receptor modulating (SERM).[11] Helix 12 of the hERs plays an important role in establishing these modes of action. It is part of the transcription activation function (AF-2) region[12], which is mainly responsible for ligand-dependent attraction of co-activators. In the full agonistic mode, the conformational equilibrium of helix 12 favors the active conformation, resulting in the attraction of co-activators.[13] Partial agonism occurs when a compound is incapable of shifting the conformational equilibrium to a fully active conformation. This results into a maximum response, which is lower than that for the full agonist E_2 or into a mixed partial agonistic/antagonistic activity. Antagonistic compounds shift the conformational equilibrium to a complete inactive conformation. Direct antagonists influence the position of helix 12 in a direct way by steric hindrance. Indirect antagonists influence the position of helix 12 by interfering with other helices of the receptor, like helices 3 or 11, or by sub-optimal contacts with the binding cavity, resulting in an antagonist orientation of helix 12.[13-15] Ultimately, this results in an impaired recruitment of co-activators. The distinction between direct and indirect antagonists can only be made with help of crystal structures of the hER and cannot be made with the results from estrogenic assays only. SERMs exhibit a tissue-specific agonistic or antagonistic mode of action. SERM behavior might be caused by differences in cofactors/corepressors, the abundance of receptor types (hERα vs hERβ), intracellular environments and intrinsic E_2 levels between the different tissues. However, the exact mechanism of which is still not fully understood.[11]

Previous research proposed a nomenclature with three different sites directing the mode of action of hERs, without accounting for the plasticity of the receptor.[16] Plasticity was probably not taken into account as the ligands used in that

research did not induce it to a large extent. As we know that upon binding of a ligand to the hERs, different helices have shown a certain degree of plasticity[17], which is thought to underlie the different modes of action of estrogenic compounds, we propose another nomenclature of the various sites contacted by ligands in the present study. Crystal structures of hERα and hERβ in complex with agonistic ligands indicated that a phenol group is important for hydrophilic interactions with amino acids in helices 3 and 6[12], here referred to as site 1. Upon binding of the agonist *ortho*-trifluoromethylphenylvinyl estradiol, a hydrophobic pocket between helix 8 and the β-sheet is formed, which is the result of the displacement of helix 7 (site 2).[17] This displacement of helix 7 does not influence the AF-2 region, thereby resulting in an agonistic mode of action. Site 3 comprises helix 12, which is displaced when a direct antagonist binds to the hERs resulting in a disordered AF-2 region.[18] Finally, site 4 comprises helix 11, which is displaced upon binding of an indirect antagonist.[13] The displacement of helix 11 influences the position of helix 12, thereby indirectly resulting in a disordered AF-2 region and consequently in an antagonistic mode of action.

Prenylation of flavonoids and isoflavonoids can modulate their estrogenic response.[19,20] Prenylation of flavones, flavanones and flavonols has been linked to an increased affinity towards hERα.[21-23] For example, chain prenylation on the 8-position of the flavanone naringenin resulted in an increased agonistic activity.[24,25] On the other hand, chain prenylation of the isoflavone genistein resulted in loss of agonistic activity.[24] It has also been suggested that prenylation of isoflavonoids can induce an antagonistic mode of action.[11,26] For example, pyran prenylation of equol together with the addition of a hydroxyl group modulated the mode of action from agonistic towards antagonistic in an hERα yeast bioassay.[27] It was not established if this antagonistic mode of action was the result of direct or indirect modulation of helix 12.

From the above it is clear that different structural features, *e.g.* flavonoids or isoflavonoid backbone structure, position and kind of prenyl, can induce different estrogenic responses *in vitro*. Hence, structure-activity relationships for these compounds need to be established further. In the present study a set of unprenylated and prenylated isoflavonoids and flavonoids were characterized on their *in vitro* estrogenic properties using a yeast estrogen bioassay. To this end, prenylated isoflavonoids and flavonoids were purified from a licorice root extract, which have never been tested for estrogenicity before. This unique set of compounds was used to obtain insight in such structure-activity relationships. Secondly, the estrogenic responses were correlated to the structural features of the receptors. The interaction between apolar, naturally occurring, prenyl substituents and hER has not been established before. Given the similarity of the prenyl substituents with the substituent of *ortho*-trifluoromethylphenylvinyl estradiol from literature, it is hypothesized that

site 2 plays a crucial in this interaction. In addition, it is hypothesized that the displacement of other helices in response to different flavonoid and isoflavonoid backbone structures or prenylation, can modulate the mode of action.

MATERIALS AND METHODS

Materials

The roots of *Glycyrrhiza glabra* were provided by Frutarom US (North Bergen, NJ, USA). An ethyl acetate extract of the roots of *Glycyrrhiza glabra*, was prepared as described previously.[27] HPLC grade acetic acid (HOAc), L-leucine, L-histidine, sodium hydroxide (NaOH) pellets, 17β-estradiol (E_2), naringenin, 6-prenylnaringenin, 8-prenylnaringenin, dehydroequol, chloroform-d_1 (99.8% atom%) and *tert*-butanol were purchased from Sigma Aldrich (St. Louis, MO, USA). Equol was purchased from Bio-connect (Huissen, The Netherlands). Glabridin was purchased from Wako (Osaka, Japan). Ethanol (analytical grade), D-(+)-glucose, ammonium sulfate and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Yeast nitrogen base without amino acids and ammonium sulphate, and agar were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). The reporter yeast strain was kindly provided by RIKILT (Wageningen, The Netherlands). ULC/MS grade acetic acid (HOAc), formic acid (FA) (ULC/MS grade), acetonitrile (ACN) (ULC/MS and HPLC-R grade), silica gel (60 Å, 70-230 mesh), water (ULC/MS grade) and methanol (ULC/MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Water for purposes other than UHPLC was prepared using a Milli-Q water purification system (Merck Millipore, Billerica, MA, USA).

RP-UHPLC-ESI-MS analysis

Samples were analyzed on an Accela ultra high pressure liquid chromatography (UHPLC) system (Thermo Scientific, San Jose, CA, USA) equipped with pump, autosampler and PDA detector. Samples (1 µL) were injected on an Acquity UPLC BEH C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity UPLC C18 Vanguard guard-column (2.1 x 5 mm, 1.7 µm particle size; Waters, Milford, MA, USA). Water acidified with 0.1% (v/v) HOAc + 1% (v/v) ACN, eluent A, and ACN acidified with 0.1% (v/v) HOAc, eluent B, were used as eluents. The flow rate was 300 µL/min, the column temperature was 35 °C and the PDA detector was set to measure a range of 200-400 nm. The following elution profile was used: 0-1 min, isocratic on 9% B; 1-22 min, linear gradient from 9-100% B; 23-27 min, isocratic on 100% B; 27-28 min, linear gradient from 100-9% B; 28-33 min, isocratic on 9% B. Mass spectrometric (MS) data was obtained by analyzing samples on a LTQ-Velos (Thermo Scientific)

equipped with a heated ESI-MS probe coupled to the RP-UHPLC. Nitrogen gas was used as sheath gas and auxiliary gas. Data was collected over an *m/z* range of 150-1500 in both positive (PI) and negative (NI) mode. Data-dependent MS^2 analysis was performed with a normalized collision energy of 35%. Settings were optimized via automatic tuning using "Tune Plus" (Xcaliber 2.2, Thermo Scientific). The system was tuned with genistein in both PI and NI modes. The source voltages were 4.50 kV and 3.50 kV, for PI and NI mode respectively. In both ionization modes the ion transfer tube temperature and the probe temperature were 400 °C and 50 °C, respectively. The identification of isoflavonoids was based on UV and MS spectra using the approach reported earlier.[28] Data acquisition and reprocessing were done with Xcalibur (version 2.2, Thermo Scientific).

Purification using RP Flash chromatography

A Grace Reveleris™ Flash system was used for pre-purification. The licorice root extract was loaded onto a 12 g Reveleris C18 RP cartridge (particle size 38.6 µm) using a solid loader (5 g cartridges) (Grace Davison Discovery Science, Deerfield, IL, USA). To this end, the licorice root extract (± 250 mg) was mixed with 1 g silica. The mixture was transferred into an empty 5 mL cartridge and closed with a plunger. The cartridge was placed upstream of the C18 RP column. Water (Milli-Q) containing 0.1% (v/v) HOAc (HPLC grade) + 1% (v/v) ACN (HPLC-R grade), eluent A, and ACN (HPLC-R grade) containing 0.1% (v/v) HOAc (HPLC-R grade), eluent B, were used as eluents. The flow rate was 30 mL/min, the following elution profile was used: 0-1.3 min, isocratic on 10% B; 1.3-2.4 min, linear gradient from 10-45% B; 2.4-21.3 min, linear gradient from 45-100% B; 21.3-24 min, isocratic on 100% B. The eluate was monitored at 280 nm and fractions (10 mL) were collected. All fractions were analyzed by UHPLC-MS as described above. Fractions containing similar prenylated flavonoids or isoflavonoids were pooled. Organic solvents from the pools were evaporated under vacuum and the remaining solution was frozen and lyophilized. The freeze-dried pools obtained were re-solubilized in 70% (v/v) aqueous ethanol for analysis by UHPLC-MS and in 100% EtOH for further purification using preparative RP-HPLC.

Gradient optimization for preparative HPLC

Purification of Flash pools into individual prenylated flavonoids and isoflavonoids was performed by a Waters preparative HPLC, equipped with a 2545 quaternary gradient module, 2767 sample manager, fluid organizer and 2998 photodiode array detector. Before the preparative purification, analytical runs were performed on the same system to optimize the gradient for elution. Gradients with different ACN slopes were

analyzed and the slope with the best peak separation was used for preparative purification. Flash pools (1 mg/mL) were injected (10 µL) on a X-bridge BEH C18 column (2.5 µm particle size, 4.6 x 30 mm, Waters) connected to a X-bridge BEH C18 column (2.5 µm particle size, 4.6 x 100 mm, Waters). In this way the analytical column has an equal resolution to the preparative column. Water acidified with 0.1% (v/v) FA, eluent A, and ACN acidified with 0.1% (v/v) FA, eluent B, were used as eluents. The flow rate was 1 mL/min and the PDA detector was set to measure a range of 200-600 nm. Mass spectrometric (MS) data were obtained by analyzing samples on a 3100 mass detector (Waters) coupled to the HPLC. Nitrogen gas was used as desolvation and cone gas. Data were collected over an *m/z* range of 200-700 in positive (PI). The system was tuned with glabridin in PI mode. For the PI mode, source temperature was 115 °C, and the capillary voltage was 3 kV. Data acquisition and reprocessing were done with Masslynx (version 4.1, Waters). The optimized analytical gradients were converted to preparative gradients using the Waters prep calculator.

Purification using preparative RP-HPLC-ESI-MS

For preparative runs, Flash pools (30 mg/mL) were injected (1.67 mL) on a X-bridge prep C18 column OBD (19 x 250 mm, 5 µm particle size, Waters). Water (MQ) acidified with 1% (v/v) FA, eluent A, and ACN (HPLC-grade) acidified with 1% (v/v) FA, eluent B, were used as eluents. "At column dilution" was used to be able to load the samples dissolved in 100% EtOH. The sample was injected into a flow of 100% ACN + 1% (v/v) FA delivered by a loading pump (HPLC pump 515, Waters). The flow rate was 1.5 mL/min for the loading pump and 15.6 mL/min for pump 2545, resulting in a total flow rate of 17.1 mL/min. The different flows were mixed via a T-piece just before the column. After the column, a small part of the flow (1:5000 splitter) was directed to the PDA detector and the MS with help of a flow of MeOH + 0.1% FA (UPLC-grade) delivered by a make-up pump (HPLC pump 515, Waters) (1 mL/min). The PDA detector was set to measure a range of 200-600 nm. For MS, nitrogen gas was used as desolvation and cone gas. Data were collected over an *m/z* range of 200-700 in positive (PI). The system was tuned with glabridin. The source temperature was 115 °C and the capillary voltage was 3 kV. Data acquisition and reprocessing were done with Masslynx (version 4.1, Waters). Fractions (5 mL) were pooled based on the response at 280 nm and MS spectra. Different gradients were used to purify the different compounds present in the different Flash pools.

The following elution profiles were used. Glabrene: 0-11.5 min, isocratic on 40% B; 11.5-50.0 min, linear gradient from 40-60% B; 50.0-53.8 min, linear gradient from 60-100% B; 53.8-69.2 min, isocratic on 100% B; 69.2-78.8 min, linear gradient from 100-40% B; 78.8-96.1 min, isocratic on 40% B.

Glabrol and 3'-hydroxy-4'-*O*-methyl-glabridin: 0-11.5 min, isocratic on 57% B; 11.5-50.0 min, linear gradient from 57-77% B; 50.0-53.8 min, linear gradient from 77-100% B; 53.8-69.2 min, isocratic on 100% B; 69.2-78.8 min, linear gradient from 100-57% B; 78.8-96.1 min, isocratic on 57% B.

4'-*O*-Methyl-glabridin: 0-11.5 min, isocratic on 60% B; 11.5-50.0 min, linear gradient from 60-70% B; 50.0-53.8 min, linear gradient from 70-100% B; 53.8-69.2 min, isocratic on 100% B; 69.2-78.8 min, linear gradient from 100-60% B; 78.8-96.1 min, isocratic on 60% B.

Hispaglabridin A: 0-11.5 min, isocratic on 60% B; 11.5-50.0 min, linear gradient from 60-75% B; 50.0-53.8 min, linear gradient from 75-100% B; 53.8-69.2 min, isocratic on 100% B; 69.2-78.8 min, linear gradient from 100-60% B; 78.8-96.1 min, isocratic on 60% B.

Hispaglabridin B: 0-11.5 min, isocratic on 70% B; 11.5-50.0 min, linear gradient from 70-85% B; 50.0-53.8 min, linear gradient from 85-100% B; 53.8-69.2 min, isocratic on 100% B; 69.2-78.8 min, linear gradient from 100-70% B; 78.8-96.1 min, isocratic on 70% B.

Solvents from the fractions were evaporated under N_2 gas, dissolved in *tert*-butanol, frozen and lyophilized. After purification the pools were analyzed using analytical UHPLC-MS.

Nuclear magnetic resonance (NMR) spectroscopy

Prior to NMR analyses, the samples were dissolved in $CDCl_3$. NMR spectra were recorded at a probe temperature of 300 °K on an Avance-III spectrometer (Bruker, Billerica, MA, USA) operating at 500 MHz located at the Wageningen NMR Centre (Wageningen, The Netherlands). To confirm the position of the methyl group of 3'-hydroxy-4'-*O*-methyl-glabridin and 4'-*O*-methyl-glabridin two-dimensional heteronuclear multiple bond correlation (2D HMBC) spectra were acquired using a standard pulse sequence delivered by Bruker. For 3'-hydroxy-4'-*O*-methyl-glabridin and 4'-*O*-methyl-glabridin 800 spectra of 104 and 24 scans, respectively, were recorded resulting in measuring times of 40 and 9 h, respectively. To confirm the position of the prenyl group on the B-ring of glabrene and hispaglabridin B, 2D HMBC and rotating frame nuclear Overhauser effect spectroscopy (ROESY) spectra were acquired using a standard pulse sequence delivered by Bruker. For both glabrene and hispaglabridin B 800 spectra of 16 scans were recorded resulting in measuring times of 8 h. Purity of the samples was based on ^1H proton spectra.

Previous ^1H and ^{13}C NMR spectra of glabrene[29], 3'-hydroxy-4'-O-methyl-glabridin[30], 4'-*O*-methyl-glabridin[30], hispaglabridin A[30], hispaglabridin B[30] and glabrol[31] confirmed our data, and 2D HMBC confirmed the positions of the methyl

group of 3'-hydroxy-4'-*O*-methyl-glabridin and 4'-*O*-methyl-glabridin. 2D HMBC and ROESY were used to indicate the position of the prenyl group on the B-ring of glabrene and hispaglabridin B. Thus, all NMR spectra confirmed the structural characteristics of the compounds purified from licorice root.

Determination of estrogenic activity

In vitro estrogenic activity was determined as described elsewhere[32] with slight modifications. Dilution series of each sample were prepared in DMSO ranging from $1x10^{-11}$ – $1x10^{-4}$ M instead of 0.0001–10 μg/mL (~$3x10^{-10}$ – $3x10^{-5}$ M). EC_{50} values and antagonistic activities were determined as described earlier.[32] If EC_{50} values could not be determined due to poor solubility or toxicity, as sometimes observed at high concentrations, agonistic activity was indicated with an EC_{50} > the highest concentration measured. The relative estrogen potency (REP) of a compound was determined by dividing the EC_{50} of E_2 by the EC_{50} of the compound. The EC_{50} values for 17β-estradiol in the ERα and ERβ bioassay were 0.70 ± 0.08 nM and 0.16 ± 0.08 nM, respectively.

Determination of the molar extinction coefficients

Molar extinction coefficients were determined using a UV-18000 spectrophotometer equipped with a CPS-controller for temperature control (Shimadzu, Kyoto, Japan). Stock solutions of 1 mg/mL in EtOH were made. Compounds were weighted on an XP6 micro-balance (Mettler-Toledo, Tiel, The Netherlands) and to minimize errors in weighing due to static interaction, an antistatic device was used (Mettler-Toledo). On the basis of stock solutions of 1 mg/mL, dilution series in EtOH were made. The absorbance at 280 nm of these dilutions were measured against EtOH in a 1 mL quarz cuvet. The temperature of the solutions was maintained at 25 °C. The molar extinction coefficients (ε) were calculated using Abs = ε * l * c, in which Abs = absorbance at 280 nm, l = light path = 1 cm, c = concentration (M). Measurements were performed with six independently prepared replicates.

In silico modelling

Molecular Operating Environment (MOE), 2013.08 (Chemical Computing Group, Montreal, QC, Canada) was used to analyze the hER 3D structures and to perform docking studies. The hERs were prepared using 3D protonate and induced fit was used as docking mode. 3D structures of the compounds were built in MOE. MOPAC energy minimization was done for all compounds, using the standard MOE software. As the exact configuration was not known for all compounds, compounds were docked in *R*- and *S*-configurations. Eventually, R-configurations were taken into

account, as this is the configuration preferred in the biosynthesis pathway and they had in general a better fit in the receptor. The protein data bank accession code used for the hERα agonist crystal structure is 3ERD[18], for the hERα agonist crystal structure with hydrophobic pocket is 2P15[33], and for the hERα direct antagonist crystal structure is 3ERT.[18] For hERβ the protein data bank accession code 4J24[34] was used for the agonist crystal structure, 1QKM for the partial agonist crystal structure[35], and 1L2J for the indirect antagonist crystal structure.[13] Using MOE, the length of the relaxed configuration of the test compounds was measured between the two most distant atoms in the molecular axis, comparable to that between 3-OH and 17-OH in E_2.

RESULTS

Purification and identification of prenylated flavonoids from licorice roots

Seven prenylated isoflavonoids and one prenylated flavonoid were identified in the licorice root extract by UHPLC-MS (Figure 3.1A), as described before.[28] Five prenylated isoflavonoids and one prenylated flavonoid were purified from the licorice root extract in a two-step purification procedure. Glabrone, the peak indicated with a star (Figure 3.1A), could not be purified and peak number 2, identified as glabridin, was not purified, as glabridin was commercially available. Figure 3.1A shows the chromatogram of the licorice root extract. As an example, the two-step purification of peak number 1 eluting at 13.7 min and identified as glabrene[28] (Table 3.1) is shown in Figures 3.1B and C. After purification by Flash chromatography (Figure 3.1B), a pool enriched in glabrene was obtained. UHPLC-UV chromatograms of all enriched Flash pools are shown in Figure S3.1. After the second purification step with preparative HPLC, >95% pure glabrene was obtained based on ^{1}H-NMR (Figure 3.1C). A similar approach was used for the five other peaks of the licorice root extract chromatogram (Figure 3.1A and Table 3.1). Besides, six compounds were purchased. Both purified and purchased compounds were analyzed with UHPLC-MS to verify the purity and to obtain their fragmentation patterns in MS^2. UHPLC-MS analysis of compound 5 (4'-*O*-methyl-glabridin) showed a purity at 280 nm of 98%, however some impurities were visible in the NI and PI mode MS spectra. For this reason, the purity of all purified compounds was based on ^{1}H-NMR (Table 3.1). Moreover, NMR analysis of all purified compounds was performed to verify the annotation made based on UHPLC-MS. The results were unambiguous and the annotation made based on UHPLC-MS was correct. Molecular extinction coefficients of compounds with a purity >90% were determined (Table 3.1). No correlation between the molecular extinction coefficients and the size

of the conjugated system was apparent. Structures of the different flavonoids and isoflavonoids are shown in Figure 3.2.

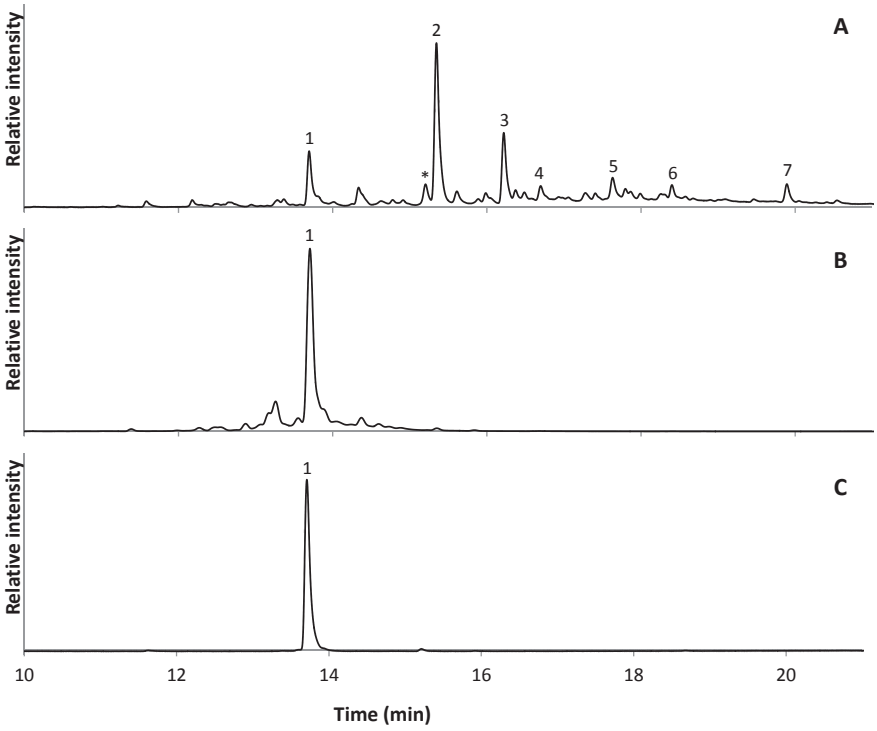

Figure 3.1. RP-UHPLC-UV profiles (280 nm) of the licorice root extract before purification (A), the pool containing glabrene after Flash chromatography (B), and glabrene after preparative HPLC (C). Peak numbers refer to compounds in Table 3.1.

Table 3.1. Overview of the spectral properties of the different flavonoids and isoflavonoids. Purified compounds are shown above the dotted line, purchased compounds are shown below.

No[a]	UV_{max} (nm)	Identification	$[M-H]^-$	MS^2 product ions (relative intensity)	$[M+H]^+$	MS^2 product ions (relative intensity)	ε_{mol}[c] ($M^{-1}. cm^{-1}$)	Purity (%)	
1	284, 296, 323	Glabrene	321	145(17), 175(23), 277(32), 293(12), 303(23), 306(100)	323	123(18), 147(40), 189(100), 213(48), 295(45)	9708 ± 470	≥95[f]	(99)[g]
3	283	Glabrol	391	187(26), 203(100)	393	203(9), 205(14), 337(100)	11748 ± 563	≥95[f]	(96)[g]
4	280	3'-Hydroxy-4'-*O*-methyl-glabridin	353	165(37), 175(27), 201(100), 338(43)	355	153(95), 189(100), 215(5)	ND	63[f]	(95)[g]
5	281	4'-*O*-Methyl-glabridin	337	149(5), 175(38), 201(100), 213(13), 322(64)	339	137(59), 189(100)	ND	81[f]	(98)[g]
6	281	Hispaglabridin A	391	177(48), 189(42), 201(52), 203(100), 215(37)	393	189(74), 191(68), 337(100)	9145 ± 321	≥95[f]	(92)[g]
7	280	Hispaglabridin B	389	175(8), 187(6), 201(100)	391	147(8), 189(100)	12254 ± 625	≥95[f]	(90)[g]
2[b]	281	Glabridin	323	121(14), 135(68), 147(27), 201(100), 213(54)	325	123(36), 189(100), 203(22), 215(7)	11175 ± 556	≥97[h]	
	243, 331	Dehydroequol	239	145(100), 197(18), 221(28), 224(53)	241	131(100), 147(44), 213(72)	ND	≥98[h]	
	281	Equol	241	119(18), 121(64), 135(23), 197(100), 223(33)	243	123(22), 199(100), 201(42), 225(62)	6761[d]	≥99[h]	
	288	Naringenin	271	151(100), 177(23)	273	147(88), 153(100)	12592 ± 631	≥95[h]	
	293, 334	6-Prenylnaringenin	339	219(100), 245(6)	341	285(100)	ND	≥95[h]	
	ND	8-Prenylnaringenin	339	219(15), 339(100)[i]	341	221(5), 285(100), 341(100)[i]	15530[e]	≥95[h]	

[a] Numbers refer to peaks in Figure 3.1.
[b] Not purified from the licorice root extract, but purchased.
[c] Measured in 100% EtOH, 25 ºC, 280 nm.
[d] Based on literature[35], measured in 96% EtOH, 281 nm.
[e] Based on literature[36], measured in MeOH, 296 nm.
[f] Based on ^{1}H NMR spectra.
[g] Based on UV 280 nm, measured with UHPLC.
[h] Based on supplier information.
[i] Based on literature[37], 8-prenylnaringenin is referred as sorphoraflavanone B.
ND Not determined.

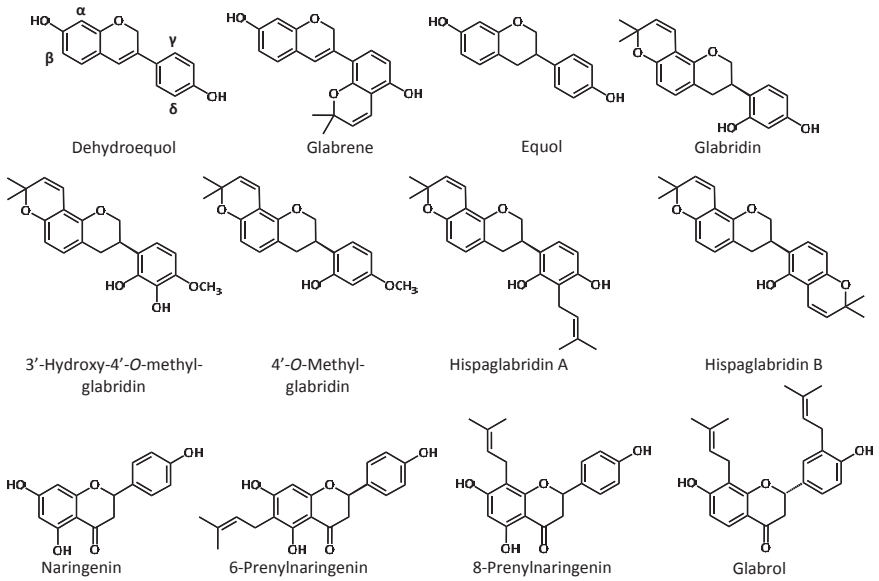

Figure 3.2. Molecular structures of the different flavonoids and isoflavonoids. Possible prenylation positions are indicated with α, β, γ and δ, as the IUPAC carbon numbering of isoflavonoids and flavonoids differs from that of pterocarpans and pterocarpenes.

Compounds with agonistic activity towards the estrogen receptors

In total, the estrogenicity of 2 isoflavenes, 6 isoflavans and 4 flavanones was determined using yeast estrogen bioassays. Table 3.2 shows the EC_{50} and REP values of the observed agonistic modes of action. REP values are indicated for comparison of the estrogenic activity measured in this studies with that in other studies, but they will not be discussed in detail. Secondly, the percentage of inhibition for observed antagonistic modes of action towards hERα and hERβ, and the concentration at which this maximum inhibition is obtained, are indicated. Clear differences in the activity between the different compounds are present. Examples of transcriptional activity via hERα in response to exposure to several compounds are shown in Figure 3.3. The agonistic activity towards hERα of equol, naringenin, 8-prenylnaringenin and the antagonistic activity towards hERα of glabridin and glabrol are shown. Moreover, it is shown that hispaglabridin A had no response towards hERα. The molecular characteristics and overall estrogenic activity of the different compounds are summarized in Table 3.3.

Table 3.2 EC_{50}, relative estrogenic potency (REP) and antagonistic response of compounds towards hERα and hERβ as obtained in yeast estrogen bioassays.

Trivial name	hERα				hERβ			
	Agonist activity		Antagonist activity		Agonistic activity		Antagonist activity	
	EC_{50} (nM)	REP[a]	Decrease (%)	Conc.[b] (nM)	EC_{50} (nM)	REP[a]	Decrease (%)	Conc.[b] (nM)
Isoflavenes								
Dehydroequol	382	$1.7x10^{-3}$	n.r.	-	3	$6.0x10^{-2}$	n.r.	-
Glabrene	>10,000[d]	$<6.6x10^{-5}$[d]	44	$1.0x10^{4}$	>10,000[d]	$<1.8x10^{-5}$[d]	63	$1.0x10^{4}$
Isoflavans								
Equol	952	$6.9x10^{-4}$	n.r.	-	43	$4.1x10^{-3}$	n.r.	-
Glabridin	n.r.	n.r.	90	$1.0x10^{4}$	n.r.	n.r.	33	$1.0x10^{4}$
3'-Hydroxy-4'-*O*-methyl-glabridin[c]	n.r.	n.r.	26	$1.0x10^{4}$	>5,000[d]	$<3.6x10^{-5}$[d]	n.r.	-
4'-*O*-Methyl-glabridin[c]	n.r.	n.r.	63	$1.0x10^{4}$	n.r.	n.r.	29	$1.0x10^{4}$
Hispaglabridin A	n.r.	n.r.	n.r.	-	n.r.	n.r.	n.r.	-
Hispaglabridin B	n.r.	n.r.	n.r.	-	>10,000[d]	$<1.8x10^{-5}$[d]	18	$1.0x10^{4}$
Flavanones								
Naringenin	>50,000[d]	$<1.3x10^{-5}$[d]	n.r.	-	2250	$8.0x10^{-5}$	n.r.	-
6-Prenylnaringenin	>5,000[d]	$<1.3x10^{-4}$[d]	n.r.	-	>500[d]	$<3.6x10^{-4}$[d]	n.r.	-
8-Prenylnaringenin	124	$5.3x10^{-3}$	n.r.	-	50	$3.6x10^{-3}$	n.r.	-
Glabrol	n.r.	n.r.	73	$5.0x10^{4}$	n.r.	n.r.	32	$5.0x10^{3}$

n.r. = No response.

[a] REP = EC_{50} of E_2 divided by the EC_{50} of the test compound. The EC_{50} value of E_2 towards hERα was 0.70 nM and towards hERβ 0.16 nM.

[b] Concentration at which the maximum decrease was reached.

[c] Purity < 90%.

[d] '>' Means that this compound does not fully reach a maximum response due to toxicity at higher concentrations and therefore the EC_{50} value cannot be determined accurately. The real EC_{50} will be higher than that given in the table. Therefore, the respective REPs are indicated by '<'.

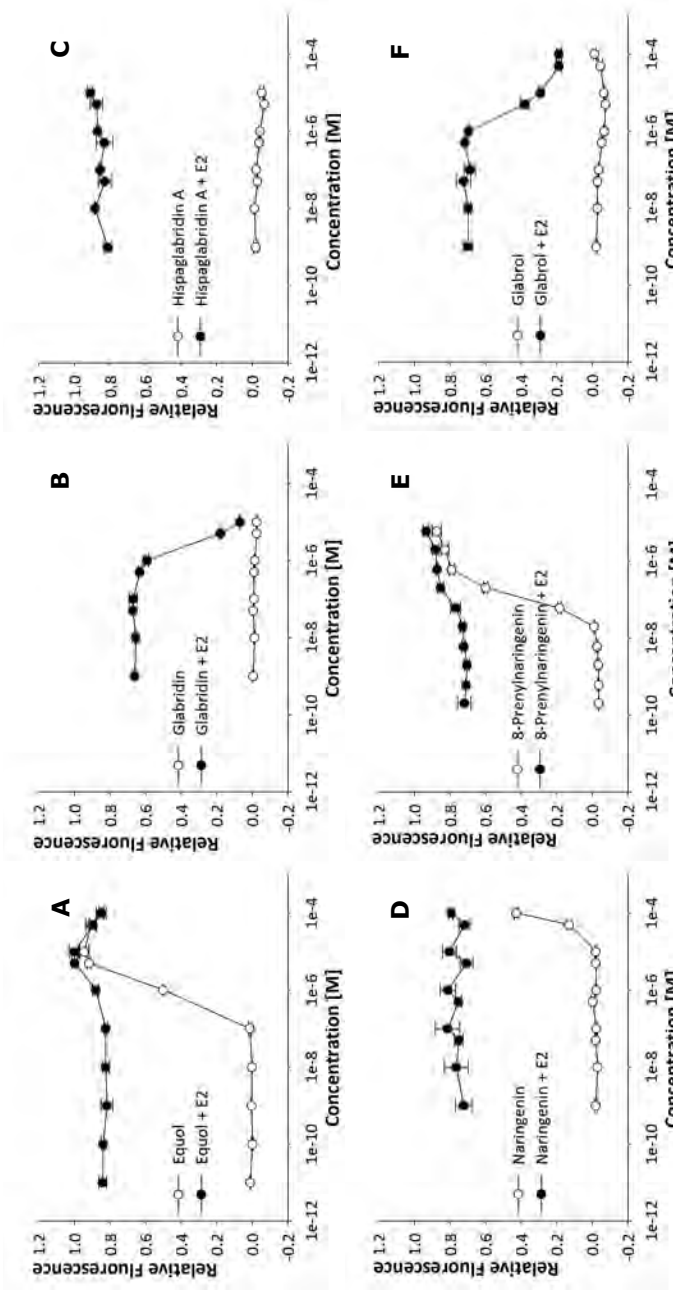

Figure 3.3. Transcriptional activity by hERα as obtained in a yeast estrogen bioassay in response to an unprenylated isoflavan (A), single prenylated isoflavan (B), double prenylated isoflavan (C), unprenylated flavanone (D), single prenylated flavanone (E), double prenylated flavanone (F).

Table 3.3. Molecular characteristics and overall activity of compounds towards hERα and hERβ as obtained in yeast estrogen bioassays.

Trivial name	Molecular characteristics				Activity		Length[b] (Å)
	1st prenyl	Pos.	2nd prenyl	Pos.	hERα	hERβ	
Isoflavenes							
Dehydroequol	-	-	-	-	Agonist	Agonist	11.97
Glabrene	Pyran	δ	-	-	Partial agonist / SERM[c]	Partial agonist / SERM[c]	11.96
Isoflavans							
Equol	-	-	-	-	Agonist	Agonist	11.97
Glabridin	Pyran	α	-	-	Antagonist	Antagonist	13.65
3′-Hydroxy-4′-*O*-methyl-glabridin[a]	Pyran	α	-	-	Antagonist	Agonist	14.41
4′-*O*-Methyl-glabridin[a]	Pyran	α	-	-	Antagonist	Antagonist	14.44
Hispaglabridin A	Pyran	α	Chain	δ	No response	No response	13.63
Hispaglabridin B	Pyran	α	Pyran	δ	No response	Partial agonist / SERM[c]	15.31
Flavanones							
Naringenin	-	-	-	-	Agonist	Agonist	10.65
6-Prenylnaringenin	Chain	β	-	-	Agonist	Agonist	10.82
8-Prenylnaringenin	Chain	α	-	-	Agonist	Agonist	10.87
Glabrol	Chain	α	Chain	γ	Antagonist	Antagonist	10.69

[a] Purity < 90%.

[b] The length of the relaxed configuration of the compounds was measured between the two most distant atoms in the molecular axis, comparable to that between 3-OH and 17-OH in E_2.

[c] Results indicate a partial agonistic or SERM activity. To classify the compounds as a SERM, different assays should give opposite mode of action, e.g. agonistic and antagonistic response.

The three unprenylated compounds, dehydroequol, equol and naringenin, showed an agonistic mode of action towards hERα and hERβ. Dehydroequol was the most active phytoestrogen of the three. Dehydroequol was more active than equol, which was expected[36,37], with EC_{50} values of 382 nM and 3 nM towards hERα and hERβ, respectively. The isoflavan equol also showed an agonistic mode of action towards both hERs. Its EC_{50} was 952 nM for hERα, which is 1.7 times lower than reported before.[38] Naringenin had an EC_{50} value of $>5.0 \times 10^4$ nM for hERα and 2250 nM for hERβ, again in line with literature.[39] All compounds showed higher activity towards hERβ than towards hERα, which is also consistent with literature.[11]

Chain prenylation of the flavanone naringenin at the 8-position, leading to 8-prenylnaringenin, increased the agonistic activity (EC_{50} hERα = 124 nM; EC_{50} hERβ = 50 nM) (Tables 3.2 and 3.3), which is in line with literature.[24] When naringenin was prenylated at the 6-position the agonistic response curve shifted also to lower concentrations, which indicated an increase in agonistic activity, but not as much as for 8-prenylnaringenin (data not shown). The EC_{50} for 6-prenylnaringenin could not be obtained, as high concentrations resulted in toxicity. Hence, the EC_{50} values for hERα and hERβ are reported as $\geq 5 \times 10^3$ nM and ≥500 nM, respectively. These data indicated that chain prenylated flavonoids have a stronger agonistic activity than unprenylated flavonoids.

Molecular docking in the agonistic crystal structures of hERα and hERβ was performed for the full agonistic compounds. It was observed that all five compounds fitted into the ligand binding cavity and similar hydrogen bonds were formed compared to those of the native ligand E_2 (His425 and Glu353 in hERα; His475 and Glu305 in hERβ; no further data shown).

Compounds with partial agonistic activity towards the estrogen receptors

Only one compound, the pyran prenylated glabrene showed partial agonistic activity towards hERα and hERβ. Pyran prenylation of the isoflavene dehydroequol, yielding glabrene, affected the estrogenic response of the compound. Whereas dehydroequol showed only agonistic activity towards both hERs, glabrene showed agonistic activity, albeit low, (EC_{50} $>1 \times 10^4$ nM towards hERα and hERβ) when tested alone, but a decrease in activity upon co-exposure with E_2 of 44% and 63% towards hERα and hERβ, respectively. This indicated that glabrene might act as partial agonist. However, it might also hint at SERM behavior, as it has been shown before that mixed activities in the yeast bioassay is indicative for SERM behavior.[40] SERMs show different modes of action in different assays and tissues, whereas partial agonists show the same mode of action. As glabrene has been shown to act as an agonist in human cell lines[27,41,42], we propose that it should be classified as a partial agonist.

Compounds with antagonistic activity towards the estrogen receptors

The mode of action towards the estrogen receptors could be modulated when isoflavonoids were pyran prenylated (Tables 3.2 and 3.3). Glabridin has an extra pyran prenyl, with a concomitant loss of the free hydroxyl group at the *C*-7 position, and an extra hydroxyl group on the *C*-2' position compared to equol. These additional substituents modulated the mode of action from full agonistic in case of equol, to antagonistic in case of glabridin. Glabridin showed antagonistic activities towards hERα and hERβ of maximum 90% and 33%, respectively (Table 3.2), which is in line with literature.[27] On the other hand, glabridin has shown to have an agonistic mode of action in human cell lines.[41,43] This implies that glabridin acts as a SERM.[43] An extra methyl group as in 4'-*O*-methyl-glabridin resulted in a small decrease in antagonistic activity towards hERα and hERβ compared to glabridin (63% and 29% signal reduction of E_2, respectively) (Tables 3.2 and 3.3). It was expected that methylation on the 4' position would decrease binding to the hERs.[44] Our results suggested that the hydroxyl group at the *C*-2' position in glabridin is also able to make hydrogen bond contacts in the hER binding cavity. Like glabridin, 4'-*O*-methyl-glabridin showed a different mode of action in human cell lines as that in our yeast bioassay.[43] This implies that 4'-*O*-methyl-glabridin also acts as a SERM. Addition of an extra hydroxyl group to form 3'-hydroxy-4'-*O*-methyl-glabridin did not change the pattern towards hERα (26% signal reduction of E_2), but it modulated the mode of action towards hERβ from antagonistic for 4'-*O*-methyl-glabridin to agonistic for 3'-hydroxy-4'-*O*-methyl-glabridin (EC_{50} > 5 x 10^3 nM) (Tables 3.2 and 3.3). This is a surprising pattern, as there are not many compounds that show an agonistic mode of action towards hERβ and an antagonistic mode of action towards hERα. So, in this case impurities might have played a role, as 3'-hydroxy-4'-*O*-methyl-glabridin had a purity of only 63%.

Single chain prenylation of the flavanone naringenin into 6- or 8-prenylnaringenin did not change the mode of action towards the hERs, whereas the double chain prenylated glabrol showed an antagonistic mode of action towards hERα and hERβ (73% and 32% signal reduction of E_2, respectively) (Tables 3.2 and 3.3). It should be mentioned that glabrol has one hydroxyl less than naringenin, which might also influence hER binding.

Compounds with no response towards the estrogen receptors

Hispaglabridin A, which has both a chain and a pyran prenyl did not show activity towards hERα or hERβ (Tables 3.2 and 3.3). Hispaglabridin B did also not show any activity towards hERα, but showed both small agonistic (EC_{50} > 1 x 10^4 nM) and antagonistic (18% signal reduction of E_2) activities towards hERβ (Tables 3.2 and 3.3). This was not expected, as *in silico* modelling studies revealed that hispaglabridin A

and B gave no reasonable fitting pose into the ligand binding cavities of hERα and hERβ agonist or antagonist structures. Previous research stated that hispaglabridin A and B could bind to estrogen receptors, but the activity was not determined.[41] In contrast, the present study shows that only hispaglabridin B has activity towards hERβ in a yeast-based cell assay, albeit relatively low.

DISCUSSION

The estrogenicity of 12 compounds was investigated using yeast based bioassays expressing human estrogen receptors. It was demonstrated that prenylation can evoke an increased agonistic activity or can change the mode of action towards the human estrogen receptors. To draw unambiguous conclusions about the impact of prenyl configuration on estrogenicity, compounds with identical backbone structures (*e.g.* isoflavan or isoflavene), but differing only in the kind of prenylation (*i.e.* chain, pyran, or furan) should be tested. It would, for example, be of interest to investigate isoflavans with a chain prenyl on the A-ring, as opposed to glabridin, to draw stronger conclusions about the influence of the prenylation of the compounds. However, such compounds have not been identified in literature. Instead, *in silico* modelling was performed in order to obtain insight in the mechanism behind the mode of action of the different subclasses of prenylated isoflavonoids and flavonoids.

Influence of the hydrophobic pocket (site 2)

In the crystal structure of hERα in complex with the full agonistic compound *ortho*-trifluoromethylphenylvinyl estradiol[33], a hydrophobic pocket, denoted as site 2, has been shown crucial in accommodating the binding of the ligand (Figure 3.4). This compound binds even more tightly to the hERα than the natural ligand E_2[45], which is probably caused by the increased number of hydrophobic contacts. Increased affinity due to extra hydrogen bonds between fluorine and the hydrophilic amino acids in the ligand binding cavity is not likely, as the fluorine atoms attached to the end of the hydrophobic chain are very weak hydrogen bond acceptors[46] (partial charge -0.34) and the distance between the fluorine atoms and the hydrophilic amino acids is quite large (3.6 Å). Chain prenylation of naringenin resulted in improved agonistic activity towards both estrogen receptors. Upon docking 8-prenylnaringenin into this crystal structure, the prenyl chain occupied the same position as the hydrophobic chain of the agonist *ortho*-trifluoromethylphenylvinyl estradiol (Figure 3.4).[17] These hydrophobic interactions at site 2 might increase the affinity of this single chain prenyl flavonoid for hER, thereby lowering its EC_{50} value. 6-Prenylnaringenin showed a moderate increase in agonistic activity compared to naringenin, but less than 8-

prenylnaringenin. The prenyl chain of 6-prenylnaringenin also interacted with the hydrophobic pocket in site 2, albeit with a less optimal fit than 8-prenylnaringenin. The S-value (docking score) for 8-prenylnaringenin (S = -38.03 kJ/mol) is in the same range as that for 6-prenylnaringenin (S = -34.56 kJ/mol), so the difference in activity cannot be explained with *in silico* docking. Crystal structures are needed to conclude about the docking position and to link this to the different activities.

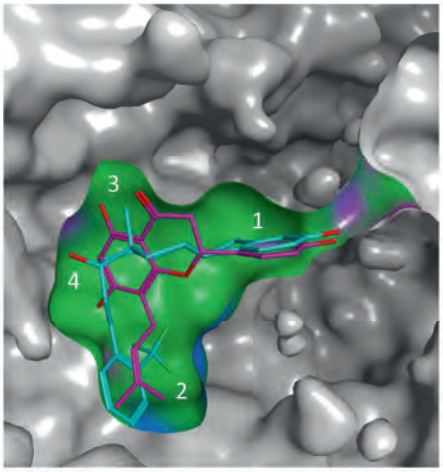

Figure 3.4. Ligand binding cavity of hERα crystalized with *ortho*-trifluoromethylphenylvinyl estradiol (cyan) (pdb: 2P15) and docked with 8-prenylnaringenin (magenta). Polar (purple), mildly polar (blue) and hydrophobic (green) areas are indicated. The different contact sites between ligand and the hER are numbered.

The antagonist glabrol, a double chain prenylated flavanone, did not give a docking solution when docked into the ligand binding cavity of the hERα and hERβ full agonist, partial agonist, direct antagonist or indirect antagonist structures. Most likely, the prenyl chain at the 8 position will fit in the hydrophobic pocket, similar to 8-prenylnaringenin, but the prenyl chain on the other side of the molecule might collide with helix 3 (site 1 of the hERs). Hence, the interaction between helices 3 and 12 might be disturbed.[15] The resulting different orientation of helix 12 will change the AF-2 co-activator region of the receptor, explaining the antagonistic mode of action observed.[47] Unfortunately, no crystal structure in which helix 3 is displaced is available to prove this hypothesis.

Influence of the moderate displacement of helix 11 (site 4)

From the estrogenicity assays, it was proposed that the pyran prenylated glabrene is a partial agonist and for that reason glabrene was docked in a partial agonistic hERβ crystal structure.[35] Glabrene fitted into the ligand binding cavity and showed similar hydrogen bond contacts compared to the partial agonistic ligand genistein. When the full agonistic crystal structure of hERβ was superimposed with the partial agonistic crystal structure of hERβ, it was observed that helix 11 was slightly shifted, combined with the outward movement of His475 (Figure 3.5).[35] In this way, the position of helix 12 and the AF-2 co-activator region is distinct from that observed in the presence of a full agonist and this will result in a less active conformation.[13,35] These observations indicate that the partial agonistic activity is characterized by the moderate displacement of helix 11 (site 4). The displacement of helix 11 will most probably be the case for the partial agonist glabrene too.

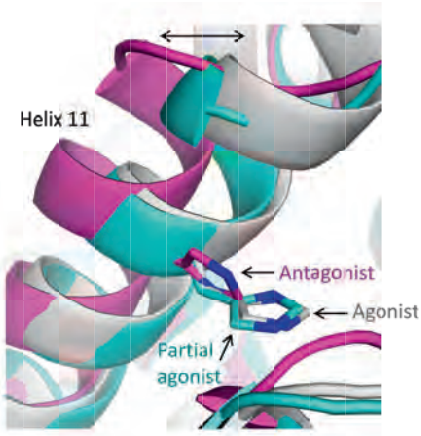

Figure 3.5. Ligand binding cavity of hERβ with helix 11 and the different orientations of His475 of the agonistic crystal structure 4J24 (grey), partial agonistic crystal structure 1QKM (cyan), and the antagonistic crystal structure 1L2J (magenta).

Influence of the extensive displacement of helix 11 (site 4)

None of the three antagonistic compounds fitted into the full agonistic or partial agonistic crystal structure of hERα and hERβ. Docking in the direct antagonistic ligand binding cavity of hERα did not result in a docking pose with a low binding energy. Docking into the indirect antagonistic ligand binding cavity of hERβ crystallized with (*R*,*R*)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (*R,R*-THC) resulted in a good fit of the three single pyran isoflavans. We suggest that single prenylated isoflavans act as indirect antagonists, by inducing an extensive shift in the position of helix 11 (site 4 of the hERa), and rotation of His475.

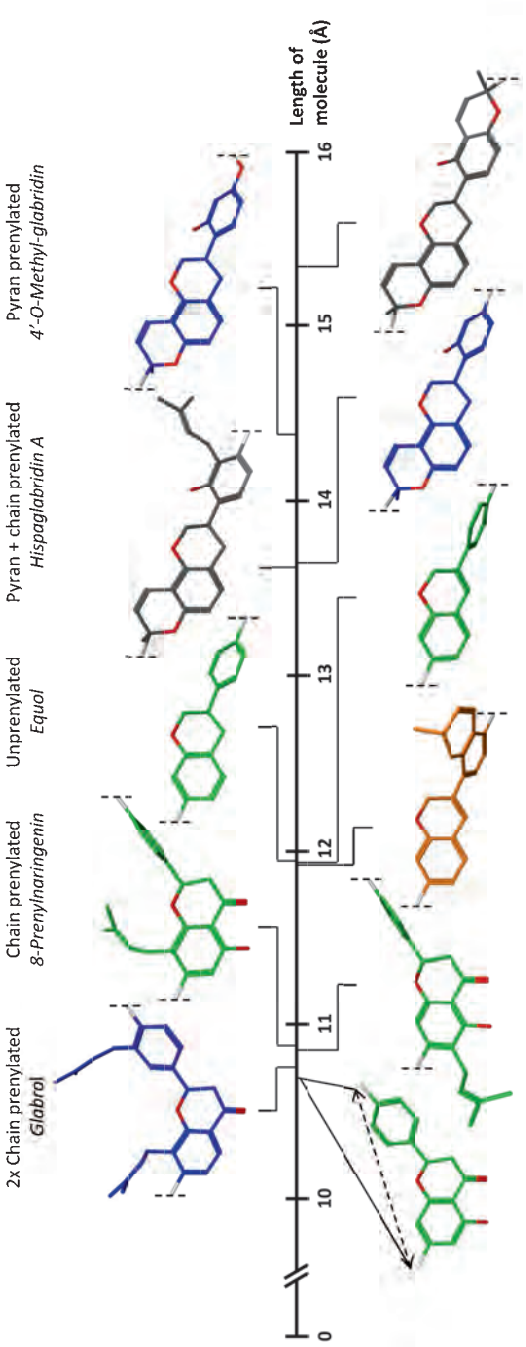

Figure 3.6. Molecular structures and length of the relaxed conformation in solution of agonists (green), partial agonists (orange), antagonists (blue) and compounds with no response (light grey). Length is measured between the atoms indicated in light grey, which are also indicated with the dotted lines. Only compounds with a purity >80% are shown. Hispaglabridin B is categorized as a compound with no response, as it showed no response towards hERα. However, hispaglabridin B showed relatively low response towards hERβ.

This is also observed when the crystal structure of the indirect antagonist *R,R*-THC is superimposed with the full agonistic crystal structure of hERβ (Figure 3.5).[13] This shift results in the destabilization of various interactions in the helix 11 region of the ligand binding cavity, yielding an inactive conformation of helix 12, which in turn triggers an antagonistic response.[13] These observations indicate that the displacement of helix 11 can be correlated to the estrogenic activity in the yeast bioassay where a moderate displacement results in partial agonistic response and an extensive displacement results in a full antagonistic response.

The hydroxyl groups on the *C*-3 and *C*-17 position of the natural full agonistic ligand E_2 are required for binding to the estrogen receptor and the distance between the groups is important for binding.[48] Prenylation of flavonoids and isoflavonoids can increase the total length of the compound. In Figure 3.6 the length of the relaxed configuration in solution and the mode of estrogenic action towards hERα are shown. Unprenylated and single prenylated compounds with a length between 10.5 and 12 Å all showed an agonistic mode of action, *e.g.* 8-prenylnaringenin and glabrene. Single prenylated compounds with a length (between 13.5 and 14.5 Å) caused an antagonistic response, which is probably caused due to their collision with helix 11. Hispaglabridin B, which has a length around 15 Å showed no response.

To conclude, chain prenylation without an increase in length resulted in an increased agonistic activity. It is hypothesized that this increase is caused by the prenyl chains, which might fit into a hydrophobic pocket present in the hER. Pyran prenylaton with an concomitant increase in length caused an indirect antagonistic response, which is suggested to be caused by collision of the pyran ring with helix 11 in the ligand binding cavity of the hER. This is the first time that estrogenic activity of prenylated flavonoids and isoflavonoids is related to structural features of the estrogen receptor.

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SUPPORTING INFORMATION

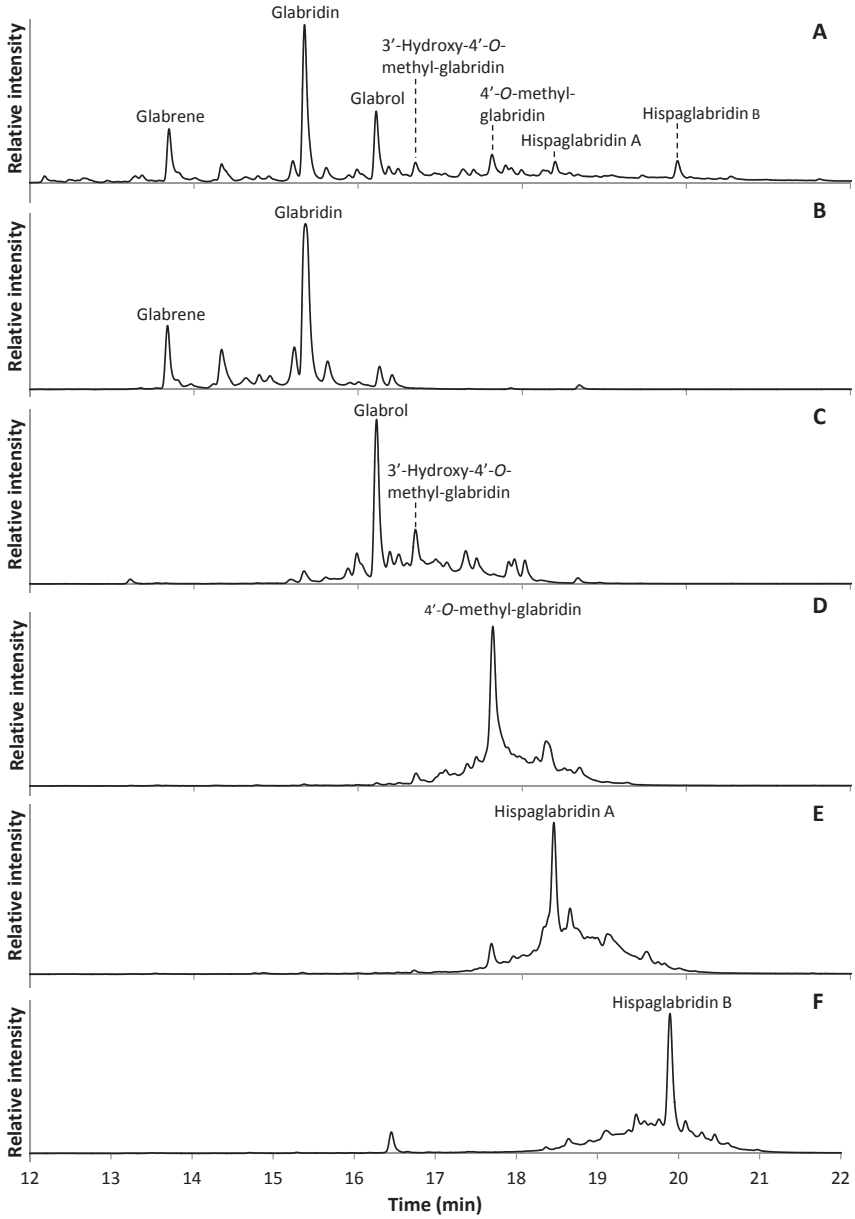

Figure S3.1. RP-UHPLC-UV profile (280 nm) of the licorice root extract (A), glabrene pool (B), glabrol and 3'-hydroxy-4'-*O*-methyl-glabridin pool (C), 4'-*O*-methyl-glabridin pool (D), hispaglabridin A pool (E), and hispaglabridin B pool (F) after Flash chromatography.

Chapter 4

Glyceollins and dehydroglyceollins isolated from soybean act as SERMs and ER subtype-selective phytoestrogens

ABSTRACT

Seven prenylated 6a-hydroxy-pterocapans and five prenylated 6a,11a-pterocarpenes with different kinds of prenylation were purified from an ethanolic extract of fungus-treated soybean sprouts. The activity of these compounds towards both human estrogen receptors (hERα and hERβ) was determined in a yeast bioassay, and the activity towards hERα was additionally tested in an U2-OS based hERα CALUX bioassay. In the yeast bioassay, compounds with chain prenylation showed in general an agonistic mode of action towards hERα, whereas furan and pyran prenylation led to an antagonistic mode of action. Five of these antagonistic compounds had an agonistic mode of action in the U2-OS based hERα CALUX bioassay, implying that these compounds can act as SERMs. The yeast bioassay also identified eight ER subtype-selective compounds, with either an antagonistic mode of action or no response towards hERα and an agonistic mode of action towards hERβ. The ER subtype-selective compounds were characterized by 6a-hydroxy-pterocarpan or 6a,11a-pterocarpene backbone structure. It is suggested that either the extra D-ring or the increase in length to 12-13.5 Å of these compounds is responsible for an agonistic mode of action towards hERβ and, thereby, inducing ER subtype-selective behavior.

Based on: Milou G.M. van de Schans, Jean-Paul Vincken, Pieter de Waard, Astrid Hamers, Toine F.H. Bovee, Harry Gruppen. ***Submitted for publication*.**

INTRODUCTION

Isoflavonoids (3-phenyl benzopyrans) are phenolic compounds, which can be found in plants of the *Leguminosae* family.[3,4] Within the *Leguminosae* family, soybean (*Glycine max*) is a rich source of isoflavonoids. The content and structural diversity of isoflavonoids can be influenced by stimulation of the plant's defense system.[1,2] The main compounds accumulating after stimulation of soybean sprouts by fungus and/or light are prenylated 6a-hydroxy-pterocarpans, the so-called glyceollins. These 6a-hydroxy-pterocarpans can be completely converted into their respective, more stable, 6a,11a-pterocarpenes after a combined acid and heat treatment (100 °C, 0.56 M HOAc, 30 min).[5] Both the 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes have structural similarities with the female sex hormone 17β-estradiol (E_2). Therefore, many of these compounds are able to interact with the human estrogen receptors (hERs), which in turn can result in adverse or beneficial health effects.[6,7]

Previous studies revealed that a mixture of prenylated 6a-hydroxy-pterocarpans had an agonistic mode of action towards hERα and hERβ in yeast estrogen bioassays[8] and showed also an agonistic mode of action in a MCF-7 based proliferation assay (E-screen).[9] However, in other studies it was shown that glyceollin I showed an antagonistic mode of action in MCF-7 cells and inhibits estrogen-induced tumor progression.[10,11] Glyceollins II and III also showed only antagonistic hER activity, although less pronounced.[10] Taken together, these data imply that the activity of glyceollins is bioassay-dependent, which indicates that glyceollins might act as selective estrogen receptor modulators (SERMs).

SERMs, like tamoxifen and raloxifene, are used for the treatment of ER-positive breast cancer. This is due to their anti-proliferative effects on breast cancer cells. This anti-proliferative effect is caused by an antagonistic mode of action towards hERα in these cancer cells.[12] However, while tamoxifen has often an antagonistic mode of action in breast cancer cells, it acts as a full agonist in the uterus, increasing the risk of developing endometrial cancer.[13] Tamoxifen and raloxifene also exhibit an agonistic mode of action in bone cells, which is regarded as beneficial as it increases bone strength in postmenopausal women.[14,15] Ever since these observations, SERMs are of great pharmaceutical interest, as they can modulate the response in one tissue differently from that in another. SERM behavior might be caused by differences in cofactors/corepressors, the abundance of receptor types (hERα vs hERβ), intracellular environments and intrinsic E_2 levels between the different tissues. ER subtype-selective compounds are also of interest, because they might also be used in the treatment of ER-positive breast cancer.[16] Theoretically, breast cancer patients should respond most positively to treatment with a compound that is an hERα-antagonist and an hERβ-agonist. Subtype-selectivity is mostly associated with differences in affinity

towards the hERs, *e.g.* compounds which exhibit an agonistic mode of action towards hERβ and (almost) no response towards hERα.[17] However, subtype-selective compounds can also show a different mode of action towards the different hERs. For example, (*R*,*R*)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (*R,R*-THC) shows an agonistic mode of action towards hERα and an antagonistic mode of action towards hERβ.[17]

Previously, it was demonstrated that the conversion of mixtures of glyceollins into mixtures of dehydroglyceollins changed the mode of action towards the estrogen receptors in yeast based estrogen bioassays.[5] Whereas glyceollins showed an agonistic mode of action towards hERα, the more planar dehydroglyceollins caused an antagonistic mode of action towards hERα. Moreover, chain prenylated naringenin showed a strong full agonistic activity, whereas genistein with the prenyl chain at the same position showed reduced estrogenic activity in MCF-7 cells.[18] This indicates that the backbone structure is an important parameter in determining estrogenicity.

Besides the backbone structure, it has been speculated that the kind of prenyl group might also influence the estrogenicity of prenylated isoflavonoids and flavonoids.[8,19] Chain prenylation of the isoflavone daidzein into kwakhurin resulted in a stronger agonistic activity compared to daidzein[20], although it should be mentioned that kwakhurin also had both an extra hydroxyl group and methyl group compared to daidzein. Pyran prenylation of daidzein led to an antagonistic mode of action for 7,8-(2,2-diMe-pyrano)daidzein.[21]

From the above it is clear that different structural features can induce different estrogenic responses *in vitro*. However, structure-activity relationships for these compounds need to be established further. In this research, first glyceollins and their corresponding dehydroglyceollins were purified from an ethanolic extract from elicited soybean seedlings. Next, their agonistic and antagonistic activity towards the hERα and hERβ were investigated using yeast estrogen bioassays and an hERα CALUX bioassay. It is hypothesized that planar backbones (as in 6a,11a-pterocarpenes) induce antagonism more efficiently than non-planar ones (as in 6a-hydroxy-pterocarpans) and that pyran prenylation induces antagonism more efficiently than chain prenylation. Moreover, it is suggested that glyceollins and dehydroglyceollins might act as SERMs and/or exhibit ER subtype-selectivity.

MATERIALS AND METHODS

Materials

Soybeans, *Glycine max* (L.) Merrill, were provided by Frutarom (Londerzeel, Belgium). An extract of germinated and *Rhizopus* challenged soybean seedlings were prepared as described previously.[2] L-leucine, L-histidine, sodium hydroxide (NaOH) pellets, chloroform-d_1 (99.8% atom%) and 17β-estradiol (E_2) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol (100%, analytical grade), D-(+)-glucose, ammonium sulfate, methanol-d_4 (99.8% atom%), acetone-d_6 (99.8% atom%) and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Yeast nitrogen base without amino acids & without ammonium sulfate, and agar were obtained from Becton-Dickinson (Franklin Lakes, NJ USA). Acetic acid (HOAc) (ULC/MS grade), acetonitrile (ACN) (ULC/MS and HPLC-R grade), silica gel (60Å, 70-230 mesh), water (ULC/MS grade) and ethyl acetate (LC-MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water for purposes other than UHPLC was prepared using a Milli-Q water purification system (Millipore, Molsheim, France). The reporter yeast strains were provided by RIKILT (Wageningen, The Netherlands) and the hERα-CALUX U2-OS cells were provided by BioDetection Systems (Amsterdam, The Netherlands).

RP-UHPLC-ESI-MS analysis

RP-UHPLC-ESI-MS analysis was performed as described previously.[5]

Purification using ethyl acetate partitioning

Six hundred mg of the soybean extract was solubilized in 70% (v/v) EtOH (10 mg/mL). Solubilization was enhanced with 5 min sonication at 25 °C. The solubilized soybean extract was added to 120 mL water, 180 mL ethyl acetate and 150 mg NaCl. This liquid-liquid partitioning was performed three times. The pooled ethyl acetate fractions were evaporated under vacuum, solubilized in *tert*-butanol and lyophilized.

Purification using RP Flash chromatography

A Grace Reveleris™ Flash system was used as first purification step. The soybean extract after ethyl acetate partitioning was injected onto a 12 g Reveleris C18 RP cartridge (particle size 38.6 µm) using a solid loader (5 g cartridges) (Grace Davison Discovery Science, Columbia, MD, USA). Therefore, the soybean extract after ethyl acetate partitioning (~250 mg) was mixed with 2 g silica. The mixture was transferred into an empty 5 mL cartridge and closed with a plunger. The cartridge was placed upstream of the C18 RP column. Water (Milli-Q) containing 1% (v/v) HOAc (HPLC

grade) + 1% (v/v) ACN (HPLC-R grade), eluent A, and ACN (HPLC-R grade) acidified with 1% (v/v) HOAc (HPLC-R grade), eluent B, were used as eluents. The flow rate was 30 mL/min and the separation was performed at room temperature. The following elution profile was used: 0-5 min, linear gradient from 0-40% B; 5-9 min, linear gradient from 40-50% B; 9-14 min, isocratic on 50% B; 14-19 min, linear gradient from 50-60% B; 19-24 min, linear gradient from 60-70% B; 24-27 min, linear gradient from 70-80% B; 27-28 min, linear gradient from 80-84% B; 28-33 min, linear gradient from 84-96% B; 33-35 min, linear gradient from 96-100% B. Absorption was measured at 280 nm. During the entire run, fractions of 10 mL were collected. All fractions were analyzed with UHPLC-MS. Fractions containing similar prenylated isoflavonoids were pooled. Next, the ACN from the pools was evaporated under vacuum at RT and the remaining water phase was immediately frozen and lyophilized in order to obtain 6a-hydroxy-pterocarpans. The pools were re-solubilized in *tert*-butanol, frozen and lyophilized in order to get a fine powder. Afterwards, the pools were re-solubilized in 70% (v/v) ethanol for analysis with UHPLC-MS and in 100% EtOH for further purification using preparative RP-HPLC.

Formation of prenylated 6a,11a-pterocarpenes

To generate prenylated 6a,11a-pterocarpenes, Flash fractions containing prenylated 6a-hydroxy-pterocarpans were evaporated to dryness. During evaporation the HOAc level increased, and 6a-hydroxy-pterocarpans are known to convert into 6a,11a-pterocarpenes under these conditions.[5] The 6a,11a-pterocarpenes were solubilized in *tert*-butanol, frozen and lyophilized. The pools were re-solubilized in 70% (v/v) ethanol for analysis with UHPLC-MS and in 100% EtOH for further purification using preparative RP-HPLC.

Gradient optimization for preparative HPLC using RP-UHPLC-ESI-MS

Before preparative purification, analytical runs were performed on the RP-UHPLC-ESI-MS system to optimize the gradient for elution. Gradients with different ACN slopes were analyzed and the slope with the best peak separation was used for preparative HPLC. Flash pools (1 mg/mL) were injected (1 µL) on a X-bridge BEH C18 column (2.1 x 75 mm, 2.5 µm particle size; Waters, Milford, MA, USA) connected to a X-bridge BEH C18 Vanguard guard-column (2.1 x 5 mm, 1.7 µm particle size; Waters). Water acidified with 1% (v/v) formic acid (FA), eluent A, and ACN acidified with 1% (v/v) FA, eluent B, were used as eluents. The flow rate was 312 µL/min, and the PDA detector was set to measure a range of 200-600 nm. Mass spectrometric (MS) data were obtained as described previously. Finally, the optimized analytical gradients

were converted to preparative gradients and “at column dilution” was taken into account.

Purification using preparative RP-HPLC-ESI-MS

Purification of Flash pools to individual prenylated isoflavonoids was performed by a Waters preparative RP-HPLC, equipped with a 2545 quaternary gradient module, 2767 sample manager, fluid organizer, 2998 photodiode array detector and 3100 mass detector. Flash pools (30 mg/mL) were injected (1.67 mL) on a X-bridge prep C18 column OBD (19 x 250 mm, 5 µm particle size, Waters). Water (MQ) acidified with 1% (v/v) FA, eluent A, and ACN (HPLC-grade) acidified with 1% (v/v) FA, eluent B, were used as eluents. “At column dilution” was used to be able to load the samples dissolved in 100% EtOH. The sample was injected in a flow of 100% ACN + 1% (v/v) FA delivered by a loading pump (HPLC pump 515, Waters). The flow rate was 1.5 mL/min for the loading pump and 15.6 mL/min for pump 2545, resulting in a total flow rate of 17.1 mL/min. The different flows were mixed via a T-piece just before the column. After the column, a small part of the flow (1:5000 splitter) was directed to the PDA detector and the MS using a flow of MeOH + 0.1% FA (UPLC-grade) delivered by a make-up pump (HPLC pump 515, Waters) (1 mL/min). The PDA detector was set to measure a range of 200-600 nm and mass spectrometric (MS) data were also obtained. For MS, nitrogen gas was used as desolvation and cone gas. Data were collected over an *m/z* range of 200-700 in positive ion mode (PI). The system was tuned with glabridin. The source temperature and the capillary voltage were 115 °C and 3 kV, respectively. Data acquisition and reprocessing were done with Masslynx (version 4.1, Waters). Fractions (5 mL) were pooled based on the response at 280 nm and MS spectra. Different gradients were used to purify the different compounds present in the different Flash pools.

The following elution profiles were used. Glyceofuran: 0-3.3 min, isocratic on 21.4% B; 3.3-59.1 min, linear gradient from 21.4-28.4% B; 59.1-62.6 min, linear gradient from 28.4-100% B; 62.6-80.0 min, isocratic on 100% B; 80.0-83.5 min, linear gradient from 100-21.4% B; 83.5-100.9 min, isocratic on 21.4% B.

Glyceollidin II: 0-3.3 min, isocratic on 30.9% B; 3.3-59.1 min, linear gradient from 30.9-37.9% B; 59.1-62.6 min, linear gradient from 37.9-100% B; 62.6-80.0 min, isocratic on 100% B; 80.0-83.5 min, linear gradient from 100-30.9% B; 83.5-100.9 min, isocratic on 30.9% B.

Glyceollin I, II and III: 0-3.3 min, isocratic on 32.0% B; 3.3-59.1 min, linear gradient from 32.0-39.0% B; 59.1-62.6 min, linear gradient from 39.0-100% B; 62.6-80.0 min, isocratic on 100% B; 80.0-83.5 min, linear gradient from 100-39.0% B; 83.5-100.9 min, isocratic on 39.0% B.

Glyceollin V: 0-3.3 min, isocratic on 38.5% B; 3.3-59.1 min, linear gradient from 38.5-45.5% B; 59.1-62.6 min, linear gradient from 45.5-100% B; 62.6-80.0 min, isocratic on 100% B; 80.0-83.5 min, linear gradient from 100-38.5% B; 83.5-100.9 min, isocratic on 38.5% B.

Glyceollin IV: 0-3.3 min, isocratic on 41.5% B; 3.3-59.1 min, linear gradient from 41.5-48.5% B; 59.1-62.6 min, linear gradient from 48.5-100% B; 62.6-80.0 min, isocratic on 100% B; 80.0-83.5 min, linear gradient from 100-41.5% B; 83.5-100.9 min, isocratic on 41.5% B.

Dehydroglyceollidin II: 0-3.3 min, isocratic on 43.0% B; 3.3-59.1 min, linear gradient from 43.0-50.0% B; 59.1-62.6 min, linear gradient from 50.0-100% B; 62.6-80.0 min, isocratic on 100% B; 80.0-83.5 min, linear gradient from 100-43.0% B; 83.5-100.9 min, isocratic on 43.0% B.

Dehydroglyceollin I, II and III: 0-3.3 min, isocratic on 52.0% B; 3.3-59.1 min, linear gradient from 52.0-59.0% B; 59.1-62.6 min, linear gradient from 59.0-100% B; 62.6-80.0 min, isocratic on 100% B; 80.0-83.5 min, linear gradient from 100-52.0% B; 83.5-100.9 min, isocratic on 52.0% B.

Dehydroglyceollin IV: 0-3.3 min, isocratic on 59.3% B; 3.3-59.1 min, linear gradient from 59.3-66.3% B; 59.1-62.6 min, linear gradient from 66.3-100% B; 62.6-80.0 min, isocratic on 100% B; 80.0-83.5 min, linear gradient from 100-59.3% B; 83.5-100.9 min, isocratic on 59.3% B.

Organic solvents from the fractions were evaporated under N_2 gas and the remaining water phase was immediately frozen and lyophilized. The compounds were re-solubilized in *tert*-butanol, frozen and lyophilized in order to get a fine powder. After purification the pools were analyzed using analytical UHPLC-MS.

Nuclear magnetic resonance (NMR) spectroscopy

Prior to NMR analyses, samples containing 6a-hydroxy-pterocarpans (glyceollins) and dehydroglyceollidin II were dissolved in methanol-d_4. Samples containing dehydroglyceollin I, II, and IV were dissolved in $CDCl_3$. The sample containing dehydroglyceollin III was dissolved in acetone-d_6. NMR spectra were recorded at a probe temperature of 300 °K on an Avance-III spectrometer (Bruker, Billerica, MA, USA) operating at 500 MHz located at the Wageningen NMR Centre (Wageningen, The Netherlands). All ^{1}H and ^{13}C NMR spectra of glyceofuran, glyceollidin II, glyceollin I, glyceollin II, glyceollin III, glyceollin IV, glyceollin V, dehydroglyceollin III were in line with previous research.[22-24]

Table 4.1. ^{1}H-NMR and ^{13}C-NMR spectral data of compounds **8, 10-12**. ^{13}C and ^{1}H-NMR chemical shifts in ppm, multiplicity, and *J* in Hz, recorded in methanol-d_4 (**8**) or $CDCl_3$ (**10-12**) at 300 °K. Chemical shifts are relative to TMS at 0.00 ppm but were actually measured to the residual solvent signals of methanol (δC = 49.00 ppm, δH = 3.310 ppm) or $CDCl_3$ (δC = 77.16 ppm, δH = 7.260 ppm).

Position	Compound 8[a]		Compound 10[a]		Compound 11[a]		Compound 12[a]	
	δC	δH, multiplicity, *J*	δC	δH, multiplicity, *J*	δC	δH, multiplicity, *J*	δC	δH, multiplicity, *J*
1	121.57	7.083, s	117.89	7.095, s	120.25	7.216, d, 8.4	121.01	7.244, s
2	122.61		115.39		109.43	6.436, d, 8.4	123.47	
3	157.25		154.57		154.08		158.40	
4	104.15	6.354, s	104.95	6.391, s	110.47		99.80	6.488, s
4A	154.28		154.96		149.40		153.41	
6	66.12	5.421, s	65.71	5.519, s	65.83	5.541, bs	65.63	5.528, s
6A	106.45		105.84		105.47		105.54	
6B	119.78		119.67		119.47		119.89	
7	119.38	7.161, d, 8.3	118.69	7.155, d, 8.3	118.60	7.134, d, 8.2	118.60	7.165, d, 8.3
8	113.08	6.731, dd, 8.3, 2.1	112.15	6.769, dd, 8.3, 1.7	112.18	6.773, m	112.06	6.769, dd, 8.3, 1.6
9	156.35		153.57		153.71		153.32	
10	99.24	6.927, d, 2.1	99.06	6.999, d, 1.7	99.09	6.997, bs	99.10	7.018, d, 1.6
10A	157.68		156.29		156.30		156.33	
11A	148.42		147.32		147.56		147.91	
11B	109.70		109.75		109.77		109.04	
12	28.46	3.235, d, 7.4	121.77	6.306, d, 9.8	116.66	6.665, d, 10.1	27.99	3.274, d, 7.1
13	124.00	5.332, m, 7.4	128.81	5.532, d, 9.8	129.91	5.625, d, 10.2	122.69	5.319, m, 7.1
14	133.06		76.91		76.37		132.62	
15	25.99	1.770, d, 1.0	28.20	1.436, s	28.00	1.443, bs	26.02	1.766, s
16	17.83	1.732, d, 0.7	28.20	1.436, s	28.00	1.443, bs	17.94	1.738, s
O-CH_3-3							55.73	3.818, s

[a] Compound numbers refer to peak numbers in Table 4.2.

s = singlet, d = doublet, dd = doublet of doublets, bs = broad singlet, m = multiplet.

Two-dimensional heteronuclear multiple bond correlation (2D HMBC) spectra were acquired using a standard pulse sequence delivered by Bruker to analyze compound 8, 10, 11 and 12 which were not reported before (Table 4.1). For compound 8, 740 spectra of 32 scans were recorded resulting in a measuring time of 12 h. For compounds 10 and 11, these values were 700 spectra of 32 scans resulting in 11.5 h. For compound 12, these values were 800 spectra of 96 scans resulting in 39 h. Purity of the samples was based on ^{1}H proton spectra. NMR unambiguously revealed that compounds 8, 10, 11 and 12 were dehydroglyceollidin II, dehydroglyceollin II, dehydroglyceollin I and dehydroglyceollin IV, respectively (Table 4.1).

Determination of estrogenic activity with a yeast bioassay

Estrogenicity was determined as described previously.[8] If EC_{50} values could not be determined due to poor solubility or toxicity, as sometimes observed at high concentrations, agonistic activity was indicated with an EC_{50} > the highest concentration measured. The relative estrogen potency (REP) of a compound was determined by dividing the EC_{50} of E_2 by the EC_{50} of the test compound. The EC_{50} values for 17β-estradiol towards hERα and hERβ in the yeast bioassay were 0.75 ± 0.04 nM and 0.24 ± 0.06 nM, respectively. The EC_{50} value for 17β-estradiol towards hERα in the CALUX bioassay was 36.7 ± 9.19 nM.

Determination of estrogenic activity with a U2-OS based hERα-CALUX bioassay

The hERα-CALUX bioassay was based on human osteoblastic osteosarcoma U2-OS cells (American Type Cell Culture) transfected with a hERα expression plasmid and a luciferase reporter construct. The hERα-CALUX bioassay was performed as described before.[25] hERα-CALUX cells were cultured in DF medium supplemented with 7.5% foetal calf serum (FCS) and 200 μg/mL G418. For exposure, hERα-CALUX cells were plated in 96-well plates with phenol red-free dermatophagoides farinae (DF) medium, supplemented with 5% (w/w) dextran-coated charcoal-stripped FCS (DCC-FCS) at a volume of 200 μL per well. Two days later, the medium was refreshed and cells were incubated with the compounds to be tested. Dilution series of each sample were prepared in DMSO ranging from $1x10^{-10}$ – $1x10^{-5}$ M. The final concentration of DMSO in the assay did not exceed 1% (v/v). Each test compound at each concentration was tested in triplicate. The samples were incubated for 24 h at 37 °C in a CO_2-incubator. After 24 h exposure, the medium was removed, cells were lysed in Triton lysis buffer, and shaken for 5 min at RT. Substrate (100 μL) containing luciferin (470 μM) was added and the luciferase activity was directly measured using a Synergy HT Multi-Detection Microplate Reader at 560 nm (BioTek Instruments, Winooski, VT, USA). For

the determination of ER antagonism, the hERα-CALUX cells were exposed to 2 µL of 6 nM or 60 nM 17β-estradiol in combination with 2 µL of different dilutions of test compound. This resulted in a final well concentration of 6 pM or 60 pM 17β-estradiol.

Determination of the molar extinction coefficient

Molar extinction coefficients were determined as described previously.[8]

In silico modelling

Molecular Operating Environment (MOE), 2013.08 (Chemical Computing Group, Montreal, QC, Canada) was used to analyze the hER 3D structures and to evaluate molecular structures. MOPAC energy minimization was done for all compounds, using the standard MOE software. The length of the relaxed configuration of the test compounds was measured between the two most distant atoms in the molecular axis, comparable to that between 3-OH and 17-OH in E_2.

RESULTS

Purification of prenylated pterocarpans and pterocarpenes

Seven prenylated 6a-hydroxy-pterocarpans and five prenylated 6a,11a-pterocarpenes were purified from an ethanolic soybean extract in a three-step purification procedure. In Figure 4.1A the RP-UHPLC-UV profile of the soybean extract is shown. Based on MS analysis it was determined that peak numbers 1 to 7 correspond to the prenylated 6a-hydroxy-pterocarpans and peaks 8 to 12 correspond to the prenylated 6a,11a-pterocarpenes (Table 4.2). Prenylated 6a,11a-pterocarpenes were only present in small amounts, as this extract was not treated with acid or heat. During the first purification step by ethyl acetate partitioning around 57% (w/w) of the extract was recovered in the ethyl acetate fraction. UHPLC-MS analysis showed that no prenylated isoflavonoids were lost in the water phase (data not shown). The second purification step was performed with Flash chromatography. Flash pools were analyzed by RP-UHPLC after Flash purification. In Figure 4.1B, the UHPLC-UV profile of the Flash pool containing peak 7, corresponding to glyceollin IV, is shown. The UHPLC-UV profile of the Flash pool containing the corresponding dehydroglyceollin IV (peak 12) is shown in Figure 4.1C. The last purification step was performed with preparative HPLC (Figures 4.1D and 4.1E) and resulted in both >95% pure glyceollin IV and dehydroglyceollin IV based on ^{1}H-NMR.

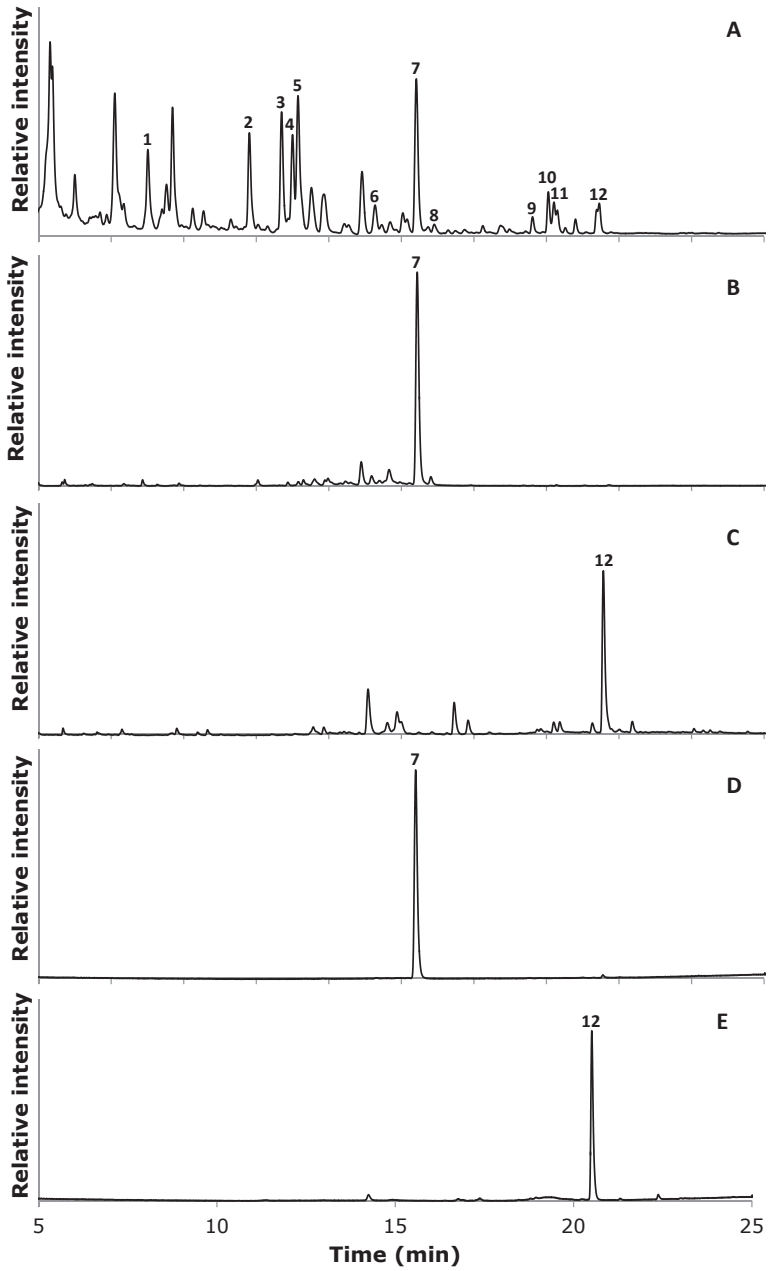

Figure 4.1. RP-UHPLC-UV profiles (280 nm) of the soybean extract before purification (A), the pool containing glyceollin IV after Flash chromatography (B), the pool containing dehdyroglyceollin IV after acid treatment of the glyceollin IV Flash pool (C), glyceollin IV (D) and dehydroglyceollin IV (E) after preparative HPLC. Peak numbers refer to compounds in Table 4.2.

Table 4.2. Overview of the spectral properties of the different prenylated isoflavonoids.

No[a]	Rt. (min)	UV_{max} (nm)	Identification	$[M-H]^-$	MS^2 product ions (relative intensity)	$[M+H]^+$	MS^2 product ions (relative intensity)	ε_{mol}[b] (M^{-1}. cm^{-1})	Purity[c] (%)
1	8.22	257, 291	Glyceofuran	353	149(19), 335(100)	337[d]	188(34), 309(100), 319(78)	4512 ± 215	95
2	10.94	285	Glyceollidin II	339	161(100), 324(58)	323[d]	276(100)	4487 ± 189	>95
3	11.81	289	Glyceollin III	337	149(13), 293(5), 319(100)	321[d]	251(90), 279(100), 306(98)	5115 ± 214	>95
4	12.07	283, 307	Glyceollin II	337	149(35), 293(24), 319(100)	321[d]	251(73), 279(100), 306(80)	6288 ± 256	92
5	12.22	283	Glyceollin I	337	149(67), 293(34), 319(100)	321[d]	293(37), 303(100), 306(84)	8634 ± 120	90
6	13.80	287, 308	Glyceollin V	335	149(28), 291(9), 317(100)	319[d]	263(100), 291(72), 319(24)	ND	>95
7	15.50	285	Glyceollin IV	353	148(5), 149(14), 335(100)	337[d]	269(69), 281(100)	7377 ± 294	>95
8	15.83	339, 355	Dehydroglyceollidin II	321	265(100), 277(10), 306(11)	323	267(100)	3994 ± 54[e]	94
9	18.73	343, 359	Dehydroglyceollin III	319	275(29), 277(100), 291(22)	321	253(30), 279(48), 293(49), 306(100)	3103 ± 190[e]	>95
10	19.19	272, 349, 364	Dehydroglyceollin II	319	275(34), 277(100), 291(66), 304(71)	321	253(19), 279(54), 293(100), 306(83)	ND	>95
11	19.36	288, 331	Dehydroglyceollin I	319	275(38), 291(100), 304(42)	321	279(18), 293(81), 306(100)	8345 ± 217	93
12	20.56	340, 356	Dehydroglyceollin IV	335	320(100)	337	281(43), 295(28), 321(100)	ND	>95

The nomenclature of glyceollins and dehydroglyceollins is ambiguous. The nomenclature in this article is based on Simons *et al.* and Aisyah *et al.*[1,2]. Glyceollidin II in this article is sometimes also referred to as glyceocarpan in the literature. Dehydroglyceollin III in this article is also referred to as glyceollin VI in the literature. In the current work, 'glyceollin' always refers to prenylated 6a-hydroxy-pterocarpans, whereas 'dehydroglyceollin' always refers to prenylated 6a,11a-pterocarpenes.

[a] Numbers refer to peaks in Figure 4.1.

[b] Measured in 100% EtOH, 25 °C, 280 nm.

[c] Based on ^{1}H proton NMR spectra.

[d] The $[M+H-H_2O]^+$ dominated in the MS^1 compared to the $[M+H]^+$.

[e] Measurement was performed with three instead of six independently prepared replications.

ND Not determined.

A similar approach was used for the 10 other peaks of the chromatogram (Figure 4.1A and Table 4.2). The purified compounds were analyzed with UHPLC-MS to obtain their fragmentation patterns in MS^2. The structure of all purified compounds was confirmed by NMR spectroscopy, as well as their purity. Molar extinction coefficients of compounds with a purity of over 90% and with a yield of over 5 mg were determined (Table 4.2).

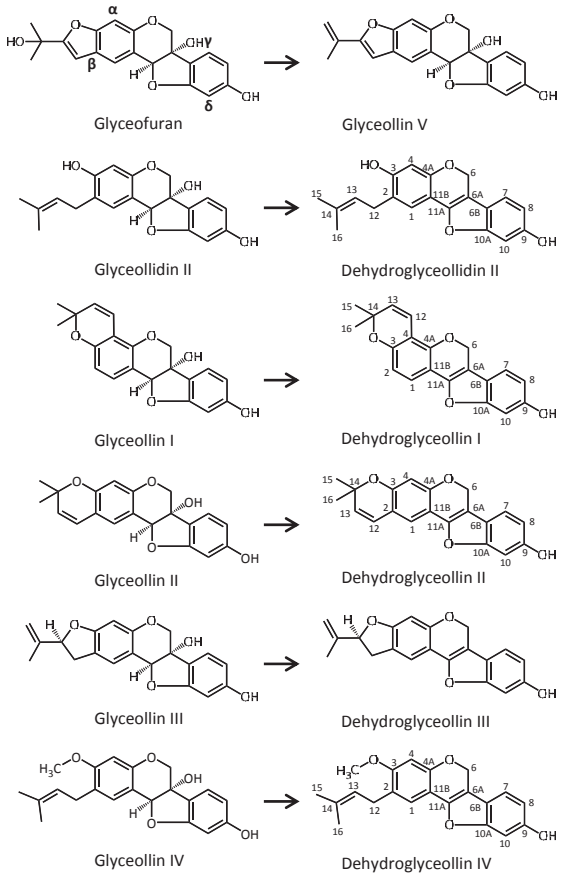

Figure 4.2. Structures of the different prenylated isoflavonoids. 6a-Hydroxy-pterocarpans are converted to the compounds indicated on the right side of the arrows as a result of high acid concentrations. Possible prenylation positions are indicated with α, β, γ and δ, as the IUPAC carbon numbering of isoflavonoids differs from that of pterocarpans and pterocarpenes. Numbering used for NMR is indicated for the structures, which were not reported before.

The molar extinction coefficients of glyceollin I, II and III were around 20% lower than reported before[26,27], but the trend was similar, with the highest molar extinction coefficient for glyceollin I and the lowest molar extinction coefficient for glyceollin III. No correlation between the molar extinction coefficients and the size of the conjugated system was apparent. Glyceollin III and glyceollidin II have higher molar extinction coefficients than their more conjugated 6a,11a-pterocarpene equivalents, which was not expected. Structures of the different isoflavonoids are shown in Figure 4.2. In this figure, it is also shown which conversions of the different 6a-hydroxy-pterocarpans occurred at elevated HOAc levels. Acid treatment of glyceofuran resulted in the formation of glyceollin V. This was not expected, as the loss of the hydroxyl group on the 6a position to form dehydroglyceofuran would result in a larger conjugated system (Figure 4.2). In previous research, a combination of heat and acid treatment was applied to glyceofuran. This resulted in a compound with MS^2 spectra different from those of glyceollin V, and that compound was annotated as dehydroglyceofuran.[5] The fact that in the present study glyceollin V was formed instead of dehydroglyceofuran, might be due to the different treatment in the present study, where only acid without concomitant heat was used.

Estrogenic properties of prenylated pterocarpans and pterocarpenes using a hERα yeast bioassay

The estrogenicity of 12 compounds was determined using a hERα yeast estrogen bioassay. Table 4.3 shows the EC_{50} values and REP values of the observed agonistic modes of action and the percentage of inhibition for the observed antagonistic modes of action towards hERα. REP values are indicated for comparison of the estrogenic activity measured in this study with those in other studies, but they will not be discussed in detail. The molecular characteristics and overall activity of the different compounds are shown in Table 4.4. Different estrogenic activities towards the hERα in yeast between the prenylated compounds were observed. The 6a-hydroxy-pterocarpans glyceofuran and glyceollidin II showed only weak agonistic activities, both with an EC_{50} above 1 x 10^5 nM. Addition of an extra methyl group to glyceollidin II to form glyceollin IV led to the complete loss of agonistic activity towards hERα, indicating that the free *C*-3 hydroxyl group is important for binding to and activating the estrogen receptor.[28] No unambiguous mode of action could be determined for dehydroglyceollin I, as it was already toxic at concentrations above 1 x 10^3 nM. Both glyceollin I and dehydroglyceollidin II showed agonistic (EC_{50} >1 x10^5 nM and >5 x10^3 nM, respectively) and antagonistic activity (32% (in mM range) and 26% (in μM range) decrease, respectively).

Table 4.3. EC_{50} concentration, relative estrogenic potency (REP) and antagonistic response of compounds towards hERα and hERβ as obtained in yeast estrogen bioassays and towards hERα as obtained in the hER CALUX bioassay.

Trivial name	Yeast hERα				Yeast hERβ				CALUX hERα		
	Agonist activity		Antagonist activity		Agonistic activity		Antagonist activity		Agonist activity		Antagonist activity
	EC_{50} (nM)	REP[a]	Decrease (%)	Conc.[b] (nM)	EC_{50}(nM)	REP[a]	Decrease (%)	Conc.[b] (nM)	EC_{50} (nM)	REP[a]	Decrease (%)
Glyceofuran	>$1.0x10^{5c}$	<$7.5x10^{-6c}$	n.r.	-	$2.6x10^{4}$	$9.2x10^{-6}$	n.r.	-	$9.1x10^{3}$	$4.0x10^{-3}$	n.r.
Glyceollidin II	>$1.0x10^{5c}$	<$7.5x10^{-6c}$	n.r.	-	$1.8x10^{4}$	$1.3x10^{-5}$	n.r.	-	$6.0x10^{3}$	$6.1x10^{-3}$	n.r.
Glyceollin I	>$1.0x10^{5c}$	<$7.5x10^{-6c}$	32	$1.0x10^{5}$	$4.4x10^{4}$	$5.5x10^{-6}$	n.r.	-	>$1.0x10^{4c}$	<$3.7x10^{-3c}$	n.r.
Glyceollin II	n.r.	n.r.	72	$1.0x10^{5}$	$2.7x10^{4}$	$8.9x10^{-6}$	n.r.	-	$5.5x10^{3}$	$6.7x10^{-3}$	n.r.
Glyceollin III	n.r.	n.r.	69	$1.0x10^{5}$	$7.0x10^{4}$	$3.4x10^{-6}$	n.r.	-	$8.5x10^{3}$	$4.3x10^{-3}$	n.r.
Glyceollin IV	n.r.	n.r.	n.r.	-	>$1.0x10^{4c}$	<$2.4x10^{-5c}$	n.r.	-	n.r.	n.r.	n.r.
Glyceollin V	n.r.	n.r.	26	$5.0x10^{4}$	$1.0x10^{4}$	$2.4x10^{-5}$	21	$1.0x10^{4}$	$4.3x10^{3}$	$8.5x10^{-3}$	n.r.
Dehydroglyceollidin II	>$5.0x10^{3c}$	<$1.5x10^{-4c}$	26	$5.0x10^{3}$	$5.0x10^{3}$	$4.8x10^{-5}$	25	$5.0x10^{3}$	$1.8x10^{3}$	$2.0x10^{-2}$	n.r.
Dehydroglyceollin I	n.r.	n.r.	n.r.	-	>$5.0x10^{3c}$	<$4.8x10^{-5c}$	15	$5.0x10^{3}$	>$5.0x10^{3c}$	<$7.3x10^{-3c}$	n.r.
Dehydroglyceollin II	n.r.	n.r.	52	$5.0x10^{3}$	>$5.0x10^{3c}$	<$4.8x10^{-5c}$	n.r.	-	>$1.0x10^{3c}$	<$3.6x10^{-2c}$	n.r.
Dehydroglyceollin III	n.r.	n.r.	27	$1.0x10^{5}$	$4.9x10^{4}$	$4.9x10^{-6}$	n.r.	-	n.r.	n.r.	n.r.
Dehydroglyceollin IV	n.r.	n.r.	50	$1.0x10^{4}$	n.r.	n.r.	n.r.	-	>$5.0x10^{3c}$	<$7.3x10^{-3c}$	n.r.

n.r. = No response.

[a] REP = EC_{50} of E_2 divided by the EC_{50} of the test compound. The EC_{50} value of E_2 towards hERα yeast was 0.75 nM, towards hERβ yeast 0.24 nM and towards hERα CALUX 36.7 nM.

[b] Concentration at which the maximum decrease was reached.

[c] '>' Means that this compound does not fully reach a maximum response due to toxicity at higher concentrations, and therefore the EC_{50} value cannot be determined accurately. The real EC_{50} will be higher than that given in the table. Therefore, REPs are indicated by '<'.

Table 4.4. Molecular characteristics, length and overall activity of compounds towards hERα and hERβ as obtained in yeast estrogen bioassays, and towards hERα as obtained in the hER CALUX bioassay.

Trivial name	Molecular characteristics		Activity			SERM	Subtype-selective	Length[a] (Å)
	Prenyl	Pos.	Yeast hERα	Yeast hERβ	CALUX hERα			
Glyceofuran	Furan	β	Agonist	Agonist	Agonist			12.76
Glyceollidin II	Chain	β	Agonist	Agonist	Agonist			10.55
Glyceollin I	Pyran	α	Partial agonist / SERM[b]	Agonist	Agonist	X	?	12.63
Glyceollin II	Pyran	β	Antagonist	Agonist	Agonist	X	X	12.60
Glyceollin III	Furan	β	Antagonist	Agonist	Agonist	X	X	12.90
Glyceollin IV	Chain	β	No response	Agonist	No response		X	11.13
Glyceollin V	Furan	β	Antagonist	Partial agonist / SERM[b]	Agonist	X	X	12.79
Dehydroglyceollidin II	Chain	β	Partial agonist / SERM[b]	Partial agonist / SERM[b]	Agonist	?	?	11.33
Dehydroglyceollin I	Pyran	α	No response	Partial agonist / SERM[b]	Agonist		X	13.17
Dehydroglyceollin II	Pyran	β	Antagonist	Agonist	Agonist	X	X	13.02
Dehydroglyceollin III	Furan	β	Antagonist	Agonist	No response		X	13.07
Dehydroglyceollin IV	Chain	β	Antagonist	No response	Agonist	X	X	12.21

[a] The length of the relaxed configuration of the compounds was measured between the two most distant atoms in the molecular axis, comparable to that between 3-OH and 17-OH in E_2.

[b] Results indicate a partial agonistic or SERM activity. To classify the compounds as a SERM, different assays should give opposite mode of action, *e.g.* agonistic and antagonistic response.

Glyceollin II, III, V and dehydroglyceollin II, III, IV all showed only antagonistic activity and no agonistic activities towards hERα with a maximum decrease of 72%, 69%, 26%, 52%, 27% and 50%, respectively. Dehydroglyceollin II showed a hERα E_2 signal reduction of 52% at the relatively low concentration of $5x10^3$ nM. The full antagonistic activity of glyceollin II towards hERα is shown in Figure 4.3A. Glyceollin II, which has pyran prenylation on the β position, resulted in a full antagonistic mode of action, whereas glyceollin I, which is pyran prenylated on the α position resulted in a combined agonistic and antagonistic response. This indicates that the position of the prenyl group is important for the estrogenic activity. Glyceollin IV did not show any activity towards hERα, whereas dehydroglyceollin IV showed only an antagonistic mode of action (Table 4.3). Both compounds have an *O*-methyl attached to their *C*-3, which was expected to strongly reduce their binding to hERα. It is known that planarity can increase the binding affinity to the estrogen receptor.[29] The removal of the 6a-hydroxyl group with the concomitant extra planarity[29] of dehydroglyceollin IV might increase the binding affinity enough to compensate for the loss in hydrogen bonding with the *C*-3 hydroxyl group.

Estrogenic properties of prenylated pterocarpans and pterocarpenes using a hERβ yeast bioassay

The estrogenicity of the 12 compounds was also determined using a hERβ yeast estrogen bioassay and the results can be found in Tables 4.3 and 4.4. Most compounds showed an agonistic mode of action towards hERβ, with EC_{50} values ranging between 5×10^3 nM and 7×10^4 nM (Table 4.3). The agonistic activity of glyceollin II towards hERβ is shown in Figure 4.3B. Addition of an *O*-methyl to the *C*-3 position of glyceollidin II to form glyceollin IV resulted in a decrease in agonistic activity. Glyceollin V, dehydroglyceollidin II and dehydroglyceollin I showed both agonistic (EC_{50} 1×10^4 nM, 5×10^3 nM and $>5 \times 10^3$ nM, respectively) and good antagonistic activity (maximum 21%, 25% and 15% decrease in the μM range, respectively). No activity was observed for dehydroglyceollin IV. None of the compounds showed only antagonistic activity.

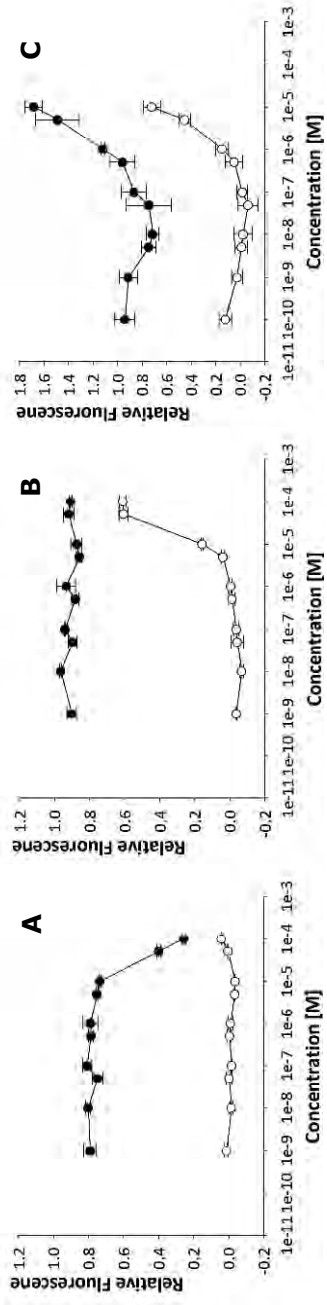

Figure 4.3. Transcriptional activity by hERα (A), hERβ (B) as obtained in a yeast estrogen bioassay, and hERα (C) as obtained in the hERα CALUX bioassay in response to the phytoSERM and ER subtype-selective glyceollin II. ♀ = Test compound (determination of agonistic activity), ♀ = Test compound + E_2 (determination of antagonistic activity).

An increase in antagonistic activity and a decrease in agonistic activity towards hERα and hERβ in the yeast bioassays was observed when prenylated 6a-hydroxy-pterocarpans were converted into their corresponding 6a,11a-pterocarpenes, except for glyceollin III. Moreover, 6a,11a-pterocarpenes displayed their activities at lower concentrations than 6a-hydroxy-pterocarpans. Between the different 6a-hydroxy-pterocarpan/6a,11a-pterocarpene sets, *e.g.* glyceollin I and dehydroglyceollin I, the lowering in concentration varied between 2- to 20-fold. This difference is probably caused by the more planar structure of dehydroglyceollins, which is suggested to increase the affinity for the estrogen receptors.[29] In our previous research, it was shown that at low concentrations, the mode of action changed from an agonistic to an antagonistic mode of action towards hERα when a mixture of glyceollins were converted into their corresponding dehydroglyceollins.[5] This was also observed at lower concentrations in the present research. So, apparently, at elevated concentrations the parental glyceollins can also exhibit antagonistic activity towards hERα.

Estrogenic properties of prenylated pterocarpans and pterocarpenes using a U2-OS based hERα CALUX bioassay

The estrogenicity of the 12 compounds was also determined using an U2-OS based hERα CALUX bioassay and the results can also be found in Tables 4.3 and 4.4. No antagonistic modes of action were observed in this hERα-CALUX bioassay. Almost all prenylated 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes showed an agonistic mode of action towards hERα in this CALUX bioassay, with EC_{50} values between 1.8 $x10^3$ and 1 $x10^4$ nM. The agonistic activity of glyceollin II towards hERα in the U2-OS cells is shown in Figure 4.3C. The kind of prenylation or backbone structure did not influence the estrogenic activity to a large extent. For glyceollin IV and dehydroglyceollin III no activities were found. Glyceollin IV is probably not able to bind to the estrogen receptor, due to the *C*-3 *O*-methyl, which hinders hydrogen bond contacts with the receptor, as discussed above.

DISCUSSION

Prerequisites for SERM behavior of (dehydro)glyceollins

An U2-OS based hERα CALUX bioassay and a hERα yeast estrogen bioassay were used to determine possible SERM behavior of the prenylated 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes towards hERα. In this way, differences in estrogenic response can only be attributed to the different cellular environments, and not to the possible presence of hERβ, as in *e.g.* normal bone cell assays. Five compounds (glyceollin II, III,

V and dehydroglyceollin II, IV) showed an antagonistic mode of action towards hERα in yeast and an agonistic mode of action towards hERα in U2-OS cells, indicating that these compounds act as SERMs. For glyceollin I and dehydroglyceollidin II it was less clear whether they could be classified as SERMs. They both showed an agonistic mode of action in the hERα-CALUX bioassay. Nevertheless, they showed agonistic activity when tested alone in the hERα yeast bioassay, but a decrease in E_2 signal upon co-exposure (antagonistic activity). This indicated that they might act as partial agonist, but it could also hint at SERM behavior if the reduction in E_2 activity upon co-exposure is truly antagonistic.[30] For glyceollin I, an hER antagonistic mode of action has already been evidenced, as it has an antagonistic mode of action in a MCF-7 cell based luciferase assay and inhibits estrogen-induced tumor progression.[10,11] In another study, glyceollin I also demonstrated an agonistic mode of action in the MCF-7 based proliferation assay (E-screen)[9] and in this research an agonistic mode of action in the hERα CALUX bioassay was also evidenced. Taken together, these data imply that glyceollin I can act as a SERM. For dehydroglyceollidin II, such additional data are not available, and consequently its classification as SERM is pending. Although SERM behavior is only observed at relative high concentrations, these compounds can be useful as natural lead molecules for the development of drugs for the treatment of breast cancer. In this research, all compounds displaying SERM behavior complied with an antagonistic mode of action towards hERα in the yeast bioassay, and an agonistic mode of action in the hERα CALUX bioassay. It should be noted that an antagonistic mode of action towards the hERα in the yeast bioassay is not typical for SERM behavior, as the opposite is possible too, *e.g.* unprenylated medicarpin, which has an agonistic mode of action in a yeast bioassay, has been classified as SERM.[31,32]

In this study, as well as in previous research[8], different modes of action towards hERα in the yeast bioassay were observed for compounds with different kind of prenylation and different backbone structures, whereas no clear differences in the mode of action towards hERα in the CALUX bioassay were observed. For that reason the estrogenicity towards the hERα in the yeast bioassay was compared between the twelve 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes from this study and the eight (iso)flavonoids from previous research.[8] The compounds tested previously consisted of two isoflavenes (dehydroequol (unprenylated) and glabrene (pyran prenylated)), three isoflavans (equol (unprenylated), glabridin and 4'-*O*-methyl-glabridin (both pyran prenylated)), and three flavanones (naringenin (unprenylated), 8-prenylnaringenin and 6-prenylnaringenin (both chain prenylated)). Only unprenylated and single prenylated flavonoids and isoflavonoids were taken into account. In this way the mode of action could be related to a single specific prenyl group. Backbone structure did not have a large influence on the mode of action towards hERα in the yeast bioassay, as different backbone structures were associated

with almost all modes of action (Figure 4.4A). When the kind of prenylation was compared, it can be observed that chain prenylation of 6a-hydroxy-pterocarpans resulted mainly in an agonistic mode of action (Figure 4.4B). Only the chain prenylated 6a,11a-pterocarpene, dehydroglyceollin IV, showed an antagonistic mode of action towards the yeast hERα. This might be caused by the increased length (+0.9 Å, Table 4.4) due to the addition of the methyl group. Furan prenylation mainly led to an antagonistic mode of action, except when it was hydroxylated, which resulted in an agonistic mode of action. In that case, the additional hydroxyl group facilitated extra hydrogen bond contacts in the ligand binding cavity of hERα when that molecule was docked in the hERα agonistic crystal structure (data not shown). This might increase the affinity towards the hERα, but apparently it also influenced the mode of action. Pyran and furan prenylation mainly resulted in an antagonistic mode of action. So, it is proposed that the kind of prenylation influences the mode of action towards hERα in the yeast bioassay and that prenylation is an important factor in determining SERM behavior.

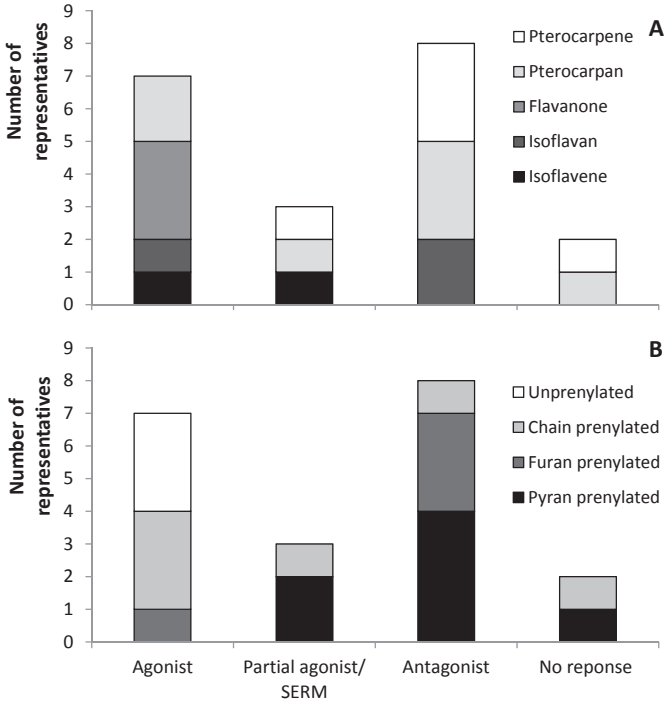

Figure 4.4. Mode of action towards hERα in the yeast bioassay related to backbone structure (A) and kind of prenylation (B).

Prerequisites for ER subtype-selective behavior of (dehydro)glyceollins

Eight isoflavonoids showed ER subtype-selective behavior, *i.e.* showing a different mode of action towards hERα and hERβ in the same cell type. Glyceollin II, III, V and dehydroglyceollin II, III showed an antagonistic mode of action towards hERα and an agonistic mode of action towards hERβ in the yeast estrogen bioassays. Glyceollin IV and dehydroglyceollin I, IV showed a response towards only one estrogen receptor. Compounds with an antagonistic mode of action towards hERα and an agonistic mode of action towards hERβ have, to the best of our knowledge, not been described before. In general, a disease like estrogen-responsive breast cancer is best treated with a compound that is an hERα antagonist and at the same time displays no or hERβ agonist activity.[16] Several of the purified compounds in this study display this pattern, although only at higher concentrations. However, with this study we do not want to imply that these compounds are direct candidates for such treatments, but the results from this research might give insight in which kind of structures cause specific hER activity profiles and are thus highly relevant for drugs designers.

In Figure 4.5 isoflavonoids and flavonoids from this study, as well as from previous research[8], as described above, were compared for their overall estrogenicity. Dehydroglyceollidin II and glyceollin I were not taken into account as their ER subtype-selective behavior was not conclusive. All ER subtype-selective compounds had an antagonistic mode of action or no response towards hERα in the yeast bioassay, which was suggested to be caused by furan or pyran prenylation (Figure 4.5B). Secondly, all ER subtype-selective compounds had an agonistic mode of action towards hERβ, except for dehydroglyceollin IV which had no response towards hERβ. Figure 4.5A shows that compounds belonging to the group of ER subtype-selective compounds consisted only of the subclasses 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes. Other subclasses studied before, like isoflavenes, isoflavans and flavanones, showed a similar pattern on both receptors[8] (Figure 4.5A). This suggests that the different mode of action towards hERβ is caused by the backbone structure rather than the kind of prenylation. Two different explanations are possible for the ER subtype-selective behavior of 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes. The extra D-ring might be the first reason, as it has been suggested that subtype-selectivity might arise from a different shape and hydrophobicity of the backbone structure.[16] The second reason is related to the length of the compounds (Figure 4.5C).

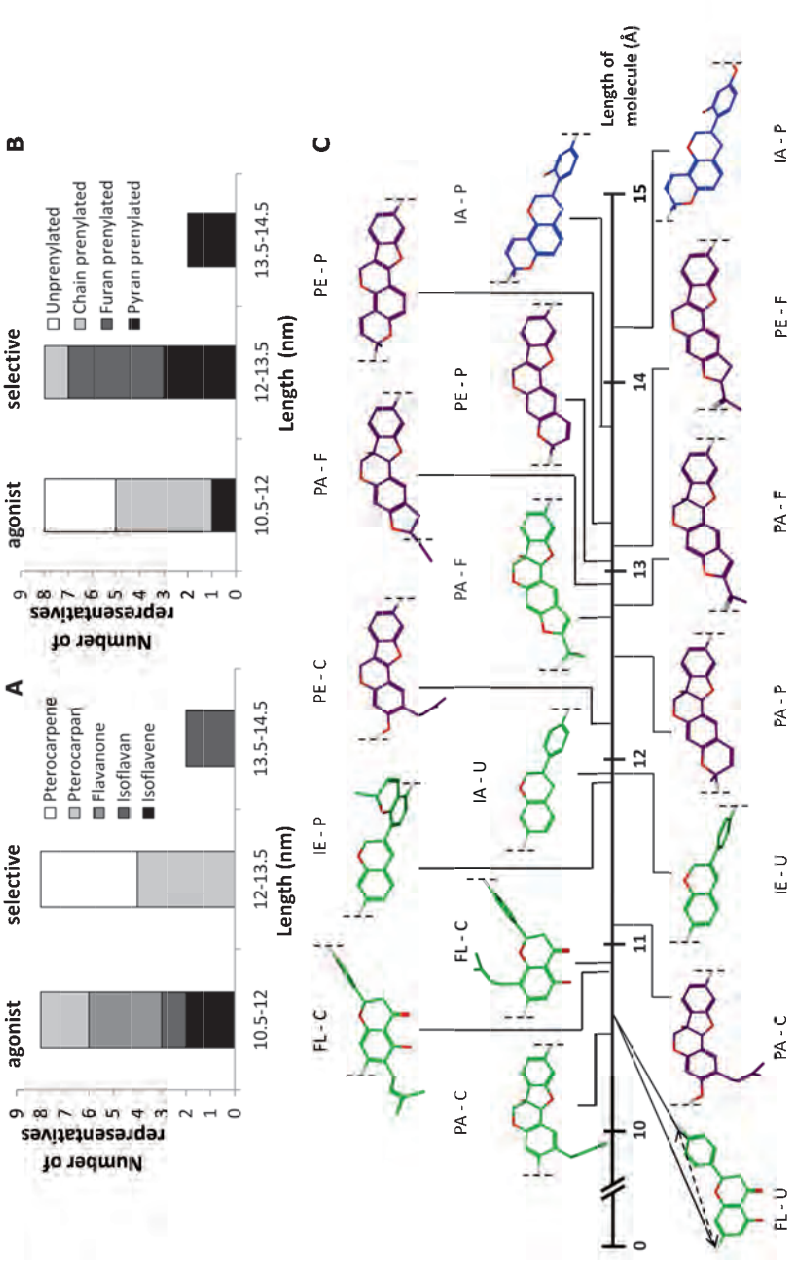

Figure 4.5. ER subtype-selective behavior related to backbone structure (A) and kind of prenylation (B). Molecular structures and length of the relaxed conformations in solution of agonists (green), ER subtype-selective compounds (purple) and antagonists (blue) (C). Length is measured between the atoms indicated in light grey, which are also indicated with the dotted lines. Only compounds with a purity >80% are shown. PA = pterocarpan, FL = flavanone, PE = pterocarpene, IA = isoflavan, IE = isoflavene, U = unprenylated, C = chain prenylated, F = furan prenylated, P = pyran prenylated.

Compounds with a length between 10.5 and 12 Å showed agonistic or partial agonistic activity towards both receptor types. Compounds with a length between 12 and 13.5 Å behaved as ER subtype-selective compounds. Compounds with a length between 13.5 and 14.5 Å showed only antagonistic activity towards both receptor types. Two exceptions, which are on the interface of agonist and ER subtype-selective compounds, should be noted: glyceofuran has the length of an ER subtype-selective compound, but showed an agonistic mode of action towards both receptor types. Secondly, glyceollin IV has the length of agonistic compounds, but showed ER subtype-selective activity. Overall, it can be concluded that the mode of action towards hERβ is influenced by backbone structure. It is suggested that the extra D-ring or the increase in length to 12-13.5 Å of the compounds is responsible for the agonistic mode of action towards hERβ and thereby inducing ER subtype-selective behavior.

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SUPPORTING INFORMATION

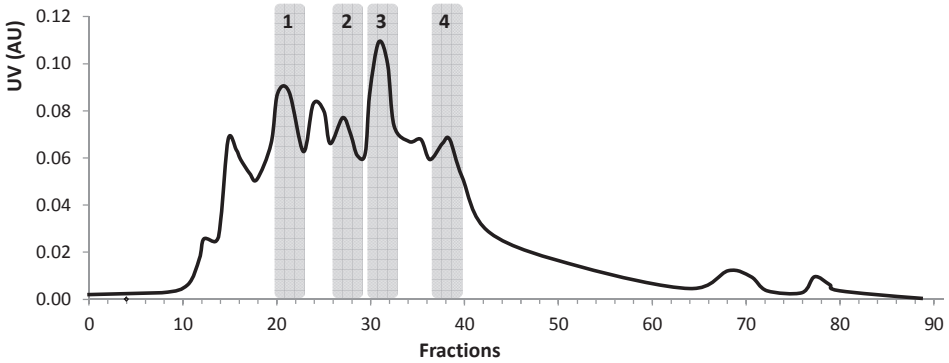


Figure S4.1. Flash chromatography UV profile (280 nm) of the untreated soybean extract. Grey areas refer to the four different pools collected. Glyceofuran (pool 1), glyceollidin II (pool 2), glyceollin I, II, III (pool 3) and glyceollin IV (pool 4).

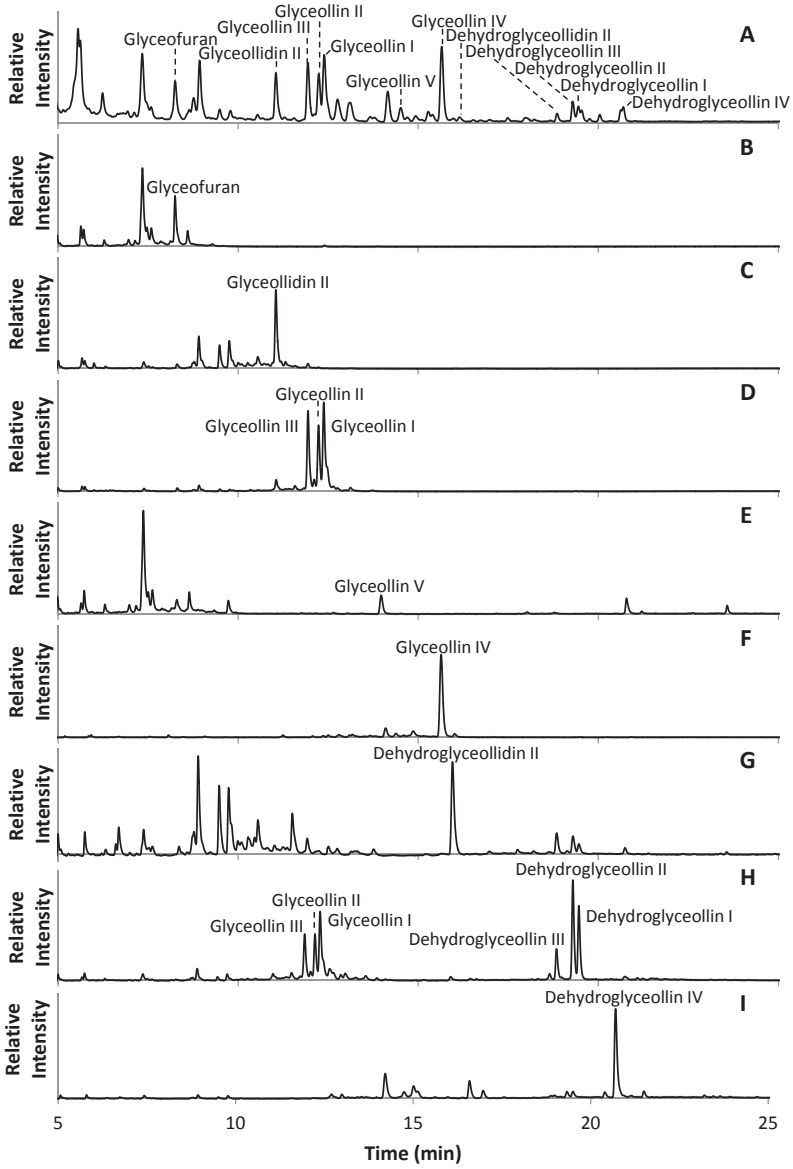


Figure S4.2. RP-UHPLC-UV profile (280 nm) of the 70% (v/v) EtOH extract from soybean seedlings elicited by fungus (A), gluceofuran pool (B), glyceollidin II pool (C), glyceollin I, II, III pool (D), glyceollin V pool (E), glyceollin IV pool (F), dehydroglyceollidin II pool (G), dehydroglyceollin I, II, III pool (H), and dehydroglyceollin IV pool (I). The various pools were obtained after Flash chromatography.

Chapter 5

Prenylation and backbone structure of flavonoids and isoflavonoids influence their phase I and II metabolism

ABSTRACT

In vitro liver metabolism of eleven prenylated flavonoids and isoflavonoids was investigated by determining their phase I glucuronyl and sulfate metabolites using pork liver preparations. Hundred metabolites were annotated using RP-UHPLC-ESI-MS^n. The positions of the hydroxyl groups attached during phase I metabolism were elucidated in-depth, considering its relevance for estrogenic activity. A mass spectrometry-based data interpretation guideline was proposed for identification of hydroxyl isomers. It was also envisioned that phase II glucuronidation is the dominant route of modification for all compounds. In order to relate structure to metabolism, compounds were classified based on three criteria: backbone structure (isoflavene, isoflavan or flavanone), number of prenyl groups (0, 1 or 2), and prenyl configuration (chain or pyran). Glucuronidation was most extensive for isoflavenes and for unprenylated compounds (yield of 90-100%). Pyran and chain prenylation gave more complex hydroxylation patterns with 4 or more than 6 hydroxyl isomers, respectively, compared to unprenylated compounds (only 1 hydroxyl isomer). Moreover, the number of hydroxyl isomers also increased with the number of prenyl groups.

Based on: Milou G.M. van de Schans, Toine F.H. Bovee, Geert M. Stoopen, Marlies Lorist, Harry Gruppen, Jean-Paul Vincken. ***Submitted for publication*.**

INTRODUCTION

Phenyl benzopyrans, comprising the subclasses of flavonoids and isoflavonoids, are secondary plant metabolites, which can be found in *Leguminosae.*[1] Flavonoids and isoflavonoids can be substituted with prenyl groups, 5-carbon substituents occurring in chain, pyran or furan configuration.[2] Both unprenylated and prenylated compounds are of interest as it has been shown that, depending on their structure, they can exhibit *in vitro* hormonal activity, *e.g.* estrogenic activity.[3-5] However, their *in vivo*, hormonal activity also depends on bioavailability and metabolism. It is known that unprenylated and prenylated flavonoids and isoflavonoids can be absorbed after ingestion.[6-8] Metabolism of estrogenic compounds has also been shown to influence the estrogenic activity.[9-12] However, metabolism of prenylated flavonoids and isoflavonoids has hardly been investigated, despite their promising bioactivities.[2]

During and after absorption of flavonoids and isoflavonoids metabolism occurs in the enterocytes and liver.[13] Two phases of metabolism are generally distinguished. Phase I metabolism leads to hydroxylation, epoxidation, hydrogenation, demethylation, removal of the sugar moiety (hydrolysis) and isomerization.[14,15] Different enzymes play a role during phase I metabolism. Nevertheless, phase I metabolism is mainly catalyzed by cytochrome P450 enzymes. During phase II metabolism glucuronic acid, sulfate, acetyl, glutathione or methyl groups are attached to the compound in order to facilitate excretion via bile and kidneys.[16] Typical enzymes in these phase II metabolic reactions are UDP-glucuronyl transferases, sulfotransferases, N-acetyl transferases, glutathione S-transferases and methyl transferases.[17] Compared to phase II, phase I metabolism is less efficient in enhancing the polarity of a compound.[18] Phase I often precedes phase II metabolism, *e.g.* hydroxylation facilitates glucuronidation, although not necessarily so.[19]

In vivo rat metabolism of one prenylated and nine unprenylated compounds from licorice was shown to result in 68 different metabolites.[15] Glucuronidation and sulfation were the major metabolic routes for the unprenylated compounds, whereas hydroxylation, glucuronidation and sulfation were the major metabolic routes for the double chain prenylated isoangustone A. The positions of the glucuronic acid, sulfate and hydroxyl substituents on isoangustone A were not determined in that study. *In vivo* metabolism of prenylated pterocarpans from soy in rat was shown to result in hydroxylation, epoxidation, glutathionylation, glucuronidation and sulfation.[20,21] It was proposed that hydroxylation occurs on the A-ring or on the prenyl group, but the exact position of the hydroxyl group was not determined. Besides attachment of different polar groups to the prenylated pterocarpans, also epoxidation of the prenyl double bond was observed in rat.[21] A similar reaction was also observed with chain-prenylated hop flavonoids using human liver microsomes. Epoxidation eventually

resulted in cyclisation of the prenyl chain, leading to metabolites with a pyran or furan ring.[22,23] None of the studies mentioned above determined structure–metabolism relationships.

As indicated above, metabolism of whole plant extracts and purified prenylated flavonoids and isoflavonoids have been investigated, but the phase I and II reactions were not studied individually and systematically. It is expected that better metabolic insights, *i.e.* more detectable metabolites, are obtained when the different phases of metabolism are performed separately *in vitro*. Therefore, the aim of the present study is to determine the *in vitro* phase I and phase II liver metabolites of 11 purified flavonoids and isoflavonoids. Pork liver preparations were used as pig is regarded as one of the best animal models for human[24] and because pork liver can easily be obtained. Moreover, differences in the number of isomers and their yield are investigated for the 11 compounds, the latter of which is not often taken into account. The metabolism of flavanones, isoflavenes and isoflavans, which are unprenylated or prenylated with one or two prenyl groups (chain and/or pyran) are compared in order to establish structure-metabolism relationships. It is hypothesized that prenylated compounds, as discussed before for isoangustone A, are more extensively hydroxylated than unprenylated compounds.

MATERIALS AND METHODS

Materials

Five prenylated isoflavonoids (glabrene, 3'-hydroxy-4'-*O*-methyl-glabridin, 4'-*O*-methyl-glabridin, hispaglabridin A and hispaglabridin B) and one prenylated flavonoid (glabrol) were purified from licorice root.[3] Naringenin, 6-prenylnaringenin, dehydroequol, magnesium chloride, bovine serum albumin (BSA), D-saccharic acid 1,4-lactone, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and uridine diphosphate glucuronic acid (UDPGA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Equol was purchased from Bio-connect (Huissen, The Netherlands) and glabridin from Wako (Osaka, Japan). Ethanol (analytical grade), disodium hydrogen phosphate, sodium dihydrogen phosphate and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Roche Diagnostics (Mannheim, Germany). DC reagents A (alkaline copper tartrate solution) and B (dilute Folin reagent) were purchased from Bio-Rad (Hercules, CA, USA). Acetic acid (HOAc) (ULC/MS grade), acetonitrile (ACN) (ULC/MS grade), water (ULC/MS grade), 0.1% (v/v) acetic acid in ACN (ULC-MS grade), 0.1% (v/v) acetic acid in water (ULC-MS grade), water/ACN 50:50 (ULC-MS grade) and methanol (ULC/MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Water for purposes other than UHPLC was prepared using a Milli-Q

water (MQ water) purification system (Merck Millipore, Billerica, MA, USA). Pork liver was obtained from butcher Henk Worst (Nijkerk, The Netherlands).

Preparation of pork S9 enzyme mixture

For the preparation of pork S9 enzyme mixture, a fresh pork liver was used, which was frozen in liquid nitrogen within 15 min after slaughter. A pig was killed with an electric shock and washed in hot water for about 1 min. The liver was cleaned with a physiological salt solution, sliced into pieces of approximately 50 g and frozen in liquid nitrogen. The tissue was mixed with 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% (w/v) KCl (ratio 1:2.5 w/v) and homogenized in a precooled blender (4 °C) for 1.5 min. The homogenate was centrifuged (9,000 x *g*, 25 min, 4 °C). After centrifugation, the supernatant (pork S9) was frozen in liquid nitrogen and stored at -80 °C.

Protein content determination of pork S9 enzyme mixture

The protein content of the pork S9 enzyme mixture was determined using a Bio-Rad DC™ protein assay. The S9 enzyme mixture was diluted 5, 10, 20, 40 and 80 times in 50 mM Tris-HCl buffer (pH 7.4, 1.15% (w/v) KCl). The assay was performed in transparent 96 wells plates with flat bottom (Greiner 655101 low protein binding). Eighteen µL of MQ water, 10 µL DC reagent A, 80 µL DC reagent B were added to 4 µL of the different concentrations of S9 enzyme mixture and mixed for 5 min at 800 rpm. The absorbance was measured after 10 min at 700 nm. The calibration curve was based on BSA with concentrations from 10 to 200 µg/mL per well (R^2=0.96). All samples were measured in triplicate.

In vitro phase I and II pork liver metabolism

Three different incubations were performed to determine the metabolite formation at different stages of metabolism: phase I metabolism, phase I and phase II metabolism and phase II metabolism with sulfation only. For phase I metabolism, 1 µL 15.4 mM test compound in 70% (v/v) EtOH, 25 µL 200 mM sodium-phosphate buffer (pH 7.4), 20 µL 25 mM $MgCl_2$ in MQ water, 4 µL 125 mM NADPH in MQ water and 25 µL S9 enzyme mix (65 mg/mL protein) were supplemented with MQ water to a total volume of 100 µL and mixed. For phase I + II metabolism, the same mix of solutions as for phase I was used, but now the mix also contained the phase II co-factors, *i.e.* 4 µL 125 nM UDPGA in MQ water, 10 µL 50 mM D-saccharic acid 1,4-lactone in MQ water and 4 µL 500 µM PAPS in 200 mM sodium-phosphate buffer (pH 8.0). To study phase II sulfation only, PAPS, but no NADPH, UDPGA and D-saccharic acid 1,4-lactone was added. All incubations were performed in duplicate and in all cases the S9 enzyme

mixture was added last. Samples without test compound, without co-factors and without S9 mixture were taken as controls. The samples were incubated in an Eppendorf incubator for 2 h at 37 °C and 500 rpm. Samples without incubation (at time point zero) were also taken for every compound. Reactions were stopped by doubling the sample volume with ice-cold acetonitrile. The samples were stored overnight at -20 °C. This set-up was tested with testosterone and the expected hydroxyl, glucuronyl and sulfate metabolites[25,26] were observed with LC-MS (method see below; data not shown).

RP-UHPLC-ESI-MS analysis of metabolites

After one night at -20 °C, the samples were centrifuged (7,000 x *g*, 15 min, 4 °C) and 180 µL supernatant was transferred to a clean vial. Next, 20 µL of 2% (v/v) HOAc and 700 µL MQ water were added, samples were mixed and loaded onto a RP-Strata X column (60 mg/3 mL, Phenomenex, Torrance, CA, USA), which was preconditioned with 2 mL MeOH and 3 mL MQ water, at a flowrate of 1 mL/min. The column was washed twice with 2 mL MQ water, after which 3 mL MeOH was used to elute the remaining compound and the formed metabolites from the column. The samples were dried under a flow of nitrogen gas at 40 °C. Samples were stored at -20 °C. Before analysis the samples were resolved in 100 µL 70% (v/v) aqueous ethanol, sonicated for 10 min and centrifuged (21,000 x *g*, 5 min, RT).

Samples were analyzed on an Accela ultra high performance liquid chromatography (RP-UHPLC) system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, auto sampler and PDA detector. Samples (8 µL) were injected on an Acquity UPLC BEH C18 column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity UPLC C18 Vanguard guard-column (2.1 x 5 mm, 1.7 µm particle size; Waters, Milford, MA, USA). Water acidified with 0.1% (v/v) HOAc + 1% (v/v) ACN, eluent A, and ACN acidified with 0.1% (v/v) HOAc, eluent B, were used as eluents. The flow rate was 300 µL/min, the column temperature was controlled at 35 °C, and the PDA detector was set to measure 200-400 nm. The following elution profile was used: 0-1 min, isocratic on 9% B; 1-22 min, linear gradient from 9-100% B; 22-24 min, isocratic on 100% B; 24-25 min, linear gradient from 100-9% B; 25-30 min, isocratic on 9% B.

Mass spectrometric (MS) data were obtained by analyzing samples on a LTQ-Velos (Thermo Scientific) equipped with a heated ESI-MS probe coupled to the RP-UHPLC. Nitrogen gas was used as sheath and auxiliary gas. Data were collected over a *m/z* range of 150-1,500 in both positive (PI) and negative (NI) mode. Data-dependent MS^2 and MS^3 analysis was performed based on dynamic exclusion with a normalized collision energy of 35%. The system was tuned with genistein in both PI and NI mode and most settings were optimized via automatic tuning using “Tune Plus” (Xcaliber

2.1.0, Thermo Scientific). For PI and NI mode MS, the source voltage was 4 kV, the ion transfer tube (ITT) temperature and the probe temperature were 400 °C and 50 °C, respectively. The identification of (iso)flavonoids was based on UV and MS spectra using the approach reported earlier.[27]. The quantification of (iso)flavonoids was performed based on their absorption at 280 nm by means of Xcalibur (version 2.1.0, Thermo Scientific). UV quantification (280 nm) of the sum of absorbances of the metabolites annotated led to an average difference of 15% in recovery compared to the decrease in absorbance of the parental compounds. Differences were expected as the molecular extinction coefficients of the metabolites formed are unknown. Besides, concentrations of metabolites below the detection limit might also influence the recovery. For those reasons, the average yield of the different metabolites was based on the decrease of absorbance of the parent compound, rather than that of the sum of metabolites.

RESULTS

Three different experimental set-ups for metabolism were performed using a freshly prepared pork liver extract and addition of different cofactors: phase I, phase I + II, and phase II sulfation only. Sulfate conjugation was not studied in combination with phase I metabolism, as NADPH added for phase I metabolism showed a decrease in sulfate conjugation (no further data shown). Sulfation was studied separately from glucuronidation, as glucuronidation was dominant over sulfation, as a result of which the full range of potentially sulfated metabolites would most likely not be formed.

After *in vitro* liver metabolism of the 11 flavonoids and isoflavonoids, 46 phase I metabolites, 42 phase I + II metabolites, and 12 sulfate metabolites were annotated using RP-UHPLC-ESI-MS (Table 5.1). As an example, Figure 5.1 shows the conversion of glabridin upon the three treatments. All expected metabolites were formed, except the epoxides of the prenyl groups. An overview of all parent compounds with putative positions of hydroxylation, glucuronidation and sulfation is shown in Figure 5.2. The annotation is discussed below.

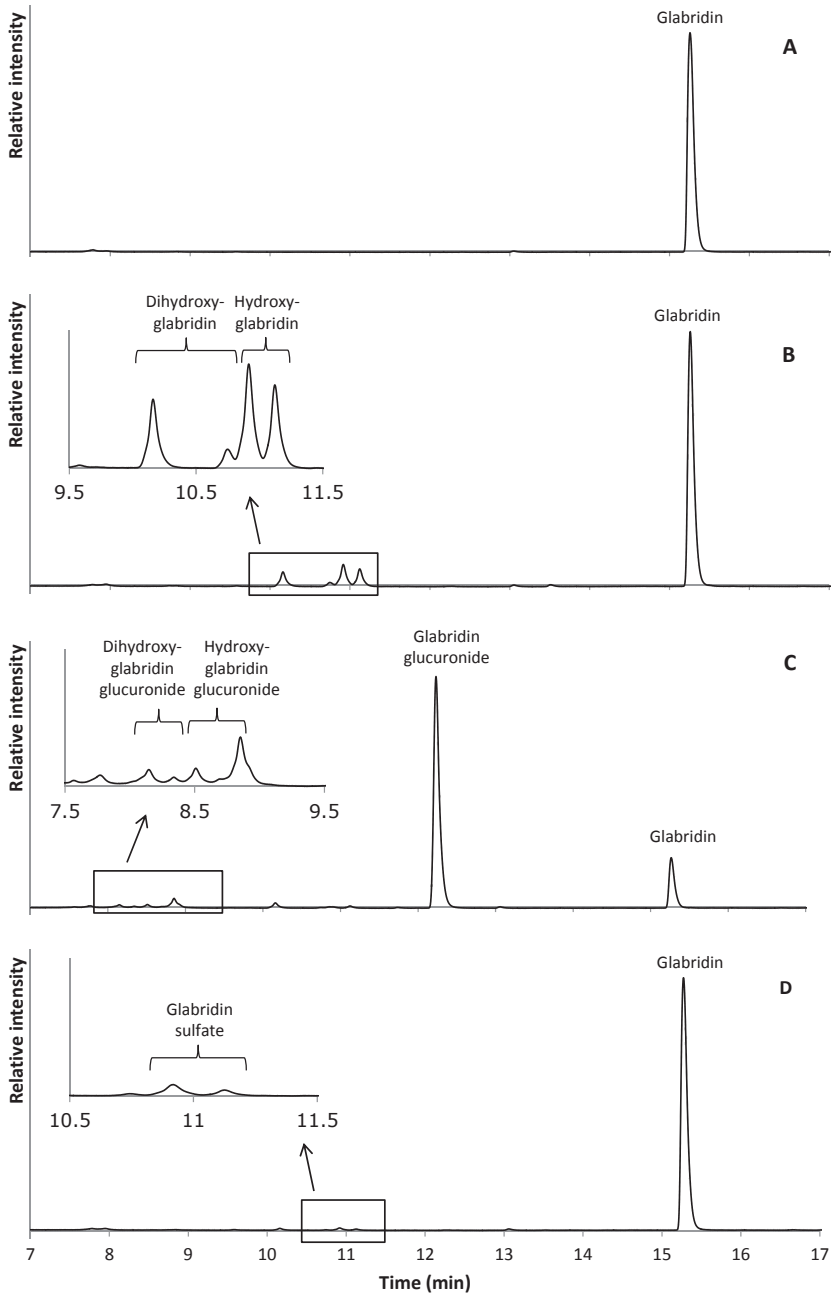


Figure 5.1. RP-UHPLC-UV profiles (280 nm) of glabridin (A), phase I metabolites of glabridin (B), phase I and II metabolites of glabridin (C), and sulfate metabolites of glabridin (D). Zoom factor of the inserts is 10x.

Table 5.1. Compounds tentatively assigned during *in vitro* metabolism of purified compounds by UHPLC-ESI-MS. U = unprenylated, C = chain prenylated, P = pyran prenylated.

Identification	RT (min)	λ_{max} (nm)	$[M-H]^-$	MS^2 product ions (relative intensity)	$[M+H]^+$	MS^2 product ions (relative intensity)	Position hydroxyl 1	Position hydroxyl 2
Isoflavenes								
Dehydroequol (U)	10.42	244, 331	239	145(100), 197(18), 221(27), 224(45)	241	131(100), 147(51), 213(66)		
Hydroxy-dehydroequol	9.11	334	255	121(3), 145(7), 211(100), 213(35), 227(38), 240(54)	257	ND	B-ring	
Dehydroequol glucuronide isomer 1	6.70	331	415	175(100), 239(20), 397(9)	417	241(100), 381(35), 399(24)		
Dehydroequol glucuronide isomer 2	7.13	241, 329	415	175(100), 239(22), 397(10)	417	241(100), 381(20), 399(13)		
Dehydroequol sulfate	7.50	333	319	239(100)	321	ND		
Glabrene (P)	13.59	284, 269, 322	321	145(15), 175(19), 277(32), 293(17), 303(28), 306(100)	323	123(10), 147(46), 189(100), 213(54), 295(42)		
Hydroxy-glabrene	12.19	284, 340	337	161(9), 293(74), 309(100), 322(58)	339	ND	A-ring	
Glabrene glucuronide isomer 1	9.68	283, 291, 323	497	175(47), 321(100), 479(8)	499	323(100), 481(10)		
Glabrene glucuronide isomer 2	10.14	283, 295, 323	497	175(100), 321(87), 479(38)	499	323(100), 481(40)		
Glabrene sulfate	9.55	285, 326	401	321(100)	403	ND		
Isoflavans								
Equol (U)	10.21	280	241	119(17), 121(100), 135(35)	243	107(48), 123(100), 137(7)		
Hydroxy-equol isomer 1	8.40	284	257	137(100), 239(5)	259	ND	A-ring	
Hydroxy-equol isomer 2	8.90	282	257	109(20), 121(62), 135(100), 147(20), 239(3)	259	ND	B-ring	
Equol-glucuronide isomer 1	7.14	279	417	175(100), 241(12), 399(31)	419	ND		
Equol-glucuronide isomer 2	7.23	278	417	175(100), 241(13), 399(34)	419	ND		
Equol sulfate	7.36	277	321	121(11), 241(100)	323	ND		

Glabridin (P)	15.26	279	323	121(37), 135(100), 147(35), 201(72), 213(41)	325	123(29), 189(100), 203(14), 215(4)		
Hydroxy-glabridin isomer 1	10.92	281	339	191(10), 203(44), 217(50), 229(20), 311(100), 321(52)	341	123(36), 205(100), 323(33)	A-ring prenyl end	
Hydroxy-glabridin isomer 2	11.12	281	339	191(10),203(43), 217(47), 229(20), 311(100), 321(50)	341	123(35), 205(100), 323(32)	A-ring prenyl end	
Dihydroxy-glabridin isomer 1[a]	10.16	283	357	135(30), 235(60), 285(100), 339(24)	341[b]	123(51), 205(100), 323(16)	A-ring prenyl (DB)	A-ring
Dihydroxy-glabridin isomer 2[a]	10.75	283	357	339(100)	341[b]	123(50), 205(100), 323(12)	A-ring prenyl (DB)	A-ring prenyl (DB)
Glabridin glucuronide	12.23	279	499	175(48), 323(100), 481(42)	501	325(100), 465(20), 483(93)		
Hydroxy-glabridin glucuronide isomer 1	8.51	280	515	175(27), 339(100), 497(30)	517	205(24), 341(68), 481(16), 499(100)	A-ring/ A-ring prenyl	
Hydroxy-glabridin glucuronide isomer 2	8.85	280	515	175(21), 339(61), 497(22)	517	205(20), 341(47), 481(30), 499(100)	A-ring/ A-ring prenyl	
Dihydroxy-glabridin glucuronide isomer 1[a]	8.15	282	533	515(100), 339(4)	517[b]	ND		
Dihydroxy-glabridin glucuronide isomer 2[a]	8.34	282	533	515(100), 339(4)	517[b]	ND		
Glabridin sulfate isomer 1	11.29	275	403	323(100)	405	ND		
Glabridin sulfate isomer 2	11.88	275	403	323(100)	405	ND		
3'-hydroxy-4'-O-methyl-glabridin (P)	16.62	279	353	165(45), 175(30), 201(100), 338(38)	355	147(3), 153(62), 189(100), 215(3)		
Dihydroxy-4'-*O*-methylglabridin isomer 1	12.03	280	369	165(18), 191(62), 203(47), 217(100), 341(49), 351(63)	371	153(70), 205(100), 353(42)	A-ring prenyl end	
Dihydroxy-4'-*O*-methylglabridin isomer 2	12.44	283	387	165(18), 191(62), 203(52), 217(100), 341(48), 351(62)	371	153(61), 205(100), 353(59)	A-ring prenyl end	
Trihydroxy-4'-*O*-methylglabridin isomer 1[a]	11.19	281	387	165(41), 235(100), 315(44), 369(33)	371[b]	153(82), 205(100), 353(10)	A-ring prenyl (DB)	A-ring
Trihydroxy-4'-*O*-methylglabridin isomer 2[a]	11.87	279	387	369(100)	371[b]	153(77), 205(100), 353(11)	A-ring prenyl (DB)	A-ring prenyl (DB)

3'-hydroxy-4'-*O*-methylglabridin glucuronide isomer 1	12.61	280	529	175(5), 353(100)	531	355(100), 513(83)		
3'-hydroxy-4'-*O*-methylglabridin glucuronide isomer 2	13.20	279	529	175(7), 353(100)	531	355(86), 513(100)		
3'-hydroxy-4'-*O*-methylglabridin sulfate	13.05	278	433	353(100)	435	355(100)		
4'-O-methyl-glabridin (P)	17.55	280	337	123(10), 149(12), 175(59), 201(100), 213(13), 322(55)	339	137(67), 147(2), 189(100), 215(4)		
Hydroxy-4'-*O*-methylglabridin isomer 1	13.17	280	353	191(15), 203(9), 217(19), 325(100), 335(16)	355	137(54), 205(100), 337(32)	A-ring prenyl end	
Hydroxy-4'-*O*-methylglabridin isomer 2	13.66	280	353	191(10), 203(8), 217(15), 325(100), 335(34)	355	137(52), 205(100), 337(42)	A-ring prenyl end	
Hydroxy-4'-*O*-methylglabridin isomer 3	14.76	283	353	165(100), 187(19), 201(15), 309(13), 321(44), 338(28)	355	147(10), 153(52), 179(46), 189(100)	B-ring	
Dihydroxy-4'-*O*-methylglabridin isomer 1[a]	12.25	283	371	149(5), 235(17), 299(100), 353(13)	355[b]	137(62), 163(20), 205(100), 337(11)	A-ring prenyl (DB)	A-ring
Dihydroxy-4'-*O*-methylglabridin isomer 2[a]	13.07	280	371	353(100)	355[b]	137(61), 163(17), 205(100), 337(11)	A-ring prenyl (DB)	A-ring prenyl (DB)
4'-*O*-methylglabridin glucuronide	13.25	279	513	175(25), 337(100), 495(22)	515	339(100), 497(80)		
Hydroxy-4'-*O*-methylglabridin glucuronide isomer 1	10.41	280	529	175(9), 353(100), 511(22)	531	285(89), 355(14), 495(41), 513(100)	A-ring/ A-ring prenyl	
Hydroxy-4'-*O*-methylglabridin glucuronide isomer 2	11.05	279	529	175(9), 353(100), 511(20)	531	285(34), 355(100), 495(13), 513(47)	A-ring/ A-ring prenyl	
Dihydroxy-4'-*O*-methylglabridin glucuronide[a]	9.41	279	547	175(13), 371(100), 529(14)	531[b]	ND		
4'-*O*-methylglabridin sulfate	12.50	272	417	337(100)	419	ND		
Hispaglabridin A (C+P)	18.31	281	391	177(56), 189(45), 201(47), 203(100), 215(38)	393	189(83), 191(78), 337(100)		
Hydroxy-hispaglabridin A isomer 1	14.58	280	407	177(9), 189(23), 203(52), 217(31), 379(100), 389(73)	409	191(57), 205(57), 353(100), 391(30)	A-ring prenyl end	

Hydroxy-hispaglabridin A isomer 2	14.86	281	407	177(9), 189(23), 203(50), 217(34), 379(100), 389(68)	409	191(60), 205(54), 353(100), 391(34)	A-ring prenyl end	
Hydroxy-hispaglabridin A isomer 3	15.19	280	407	193(5), 201(8), 205(9), 219(16), 377(100), 389(21)	409	189(11), 207(10), 353(100), 391(42)	B-ring	
Hydroxy-hispaglabridin A isomer 4	16.04	281	407	193(5), 201(9), 205(9), 219(17), 377(100), 389(57)	409	189(10), 207(6), 391(100)	B-ring prenyl	
Hydroxy-hispaglabridin A isomer 5	16.52	280	407	193(6), 201(12), 205(13), 219(25), 377(100), 389(59)	409	189(16), 207(12), 391(100)	B-ring prenyl	
Hydroxy-hispaglabridin A isomer 6	16.86	281	407	193(10), 201(66), 219(29), 349(100), 389(23)	409	189(33), 207(10), 353(13), 391(42)	B-ring/ B-ring prenyl	
Dihydroxy-hispaglabridin A isomer 1[a]	13.75	280	425	177(19), 203(48), 215(3), 235(35), 353(100), 407(29)	409[b]	191(27), 205(29), 353(100), 391(4)	A-ring prenyl (DB)	A-ring
Dihydroxy-hispaglabridin A isomer 2[a]	13.98	280	425	407(100)	409[b]	191(27), 205(28), 353(100), 391(4)	A-ring prenyl (DB)	A-ring prenyl (DB)
Dihydroxy-hispaglabridin A isomer 3	13.30	280	423	203(34), 219(4), 351(100), 365(14), 381(15), 405(6)	425	ND	A-ring/ A-ring prenyl	A-ring/ A-ring prenyl
Hispaglabridin A glucuronide	14.38	279	567	175(18), 391(100), 549(36)	569	393(100), 513(43), 551(63)		
Hydroxy-hispaglabridin A glucuronide isomer 1	10.50	280	583	175(6), 407(100), 565(30)	585	409(81), 503(100), 525(39), 567(68)	A-ring prenyl end	
Hydroxy-hispaglabridin A glucuronide isomer 2	11.30	280	583	175(8), 407(100), 565(31)	585	409(100), 503(36), 525(57), 567(33)	A-ring prenyl end	
Hydroxy-hispaglabridin A glucuronide isomer 3	11.83	280	583	175(3), 407(100), 565(21)	585	409(100), 503(18), 567(11)	B-ring/ B-ring prenyl	
Hydroxy-hispaglabridin A glucuronide isomer 4	12.20	279	583	175(7), 407(60), 565(100)	585	409(100), 503(58), 567(75)	B-ring	
Hydroxy-hispaglabridin A glucuronide isomer 5	12.75	280	583	175(13), 407(97), 501(100), 565(23)	585	409(100), 503(49), 567(20)	B-ring/ B-ring prenyl	

Hydroxy-hispaglabridin A glucuronide isomer 6	13.10	275	583	175(4), 407(100), 565(14)	585	409(100), 503(28), 567(34)	B-ring/ B-ring prenyl	
Dihydroxy-hispaglabridin A glucuronide isomer 1[a]	10.33	276	601	583(100)	585[b]	409(43), 503(100), 567(34)	A-ring prenyl (DB)	A-ring/ A-ring prenyl
Hispaglabridin B (P + P)	19.79	279	389	175(16), 187(8), 201(100)	391	147(7), 189(100)		
Hydroxy-hispaglabridin B isomer 1	15.28	280	405	175(6), 187(11), 201(58), 203(44), 217(100)	407	189(100), 205(97), 351(41), 389(89)	UNK	
Hydroxy-hispaglabridin B isomer 2	16.00	280	405	175(6), 187(11), 201(65), 203(44), 217(100)	407	189(93), 205(100), 351(45), 389(95)	UNK	
Dihydroxy-hispaglabridin B isomer 1[a]	14.65	278	423	175(18), 201(100), 235(95), 351(20), 405(10)	407[b]	189(90), 205(100), 351(4), 389(7)	UNK	UNK
Dihydroxy-hispaglabridin B isomer 2[a]	14.89	278	423	175(11), 201(64), 235(42), 351(26), 405(100)	407[b]	189(61), 205(100), 351(8), 389(6)	UNK	UNK
Hispaglabridin B glucuronide	15.58	278	565	175(34), 389(100), 547(11)	567	391(100), 549(67)		
Hydroxy-hispaglabridin B glucuronide isomer 1	11.18	278	581	175(20), 405(100), 563(15)	583	ND	UNK	
Hydroxy-hispaglabridin B glucuronide isomer 2	12.16	278	581	175(20), 405(100), 563(17)	583	ND	UNK	
Dihydroxy-hispaglabridin B glucuronide[a]	11.06	278	599	175(9), 405(9), 423(81), 581(100)	583[b]	407(100), 565(73)	UNK	UNK
Hispaglabridin B sulfate	14.64	280	469	389(100)	471	ND		
Flavanones								
Naringenin (U)	10.08	288	271	151(100), 177(23)	273	147(86), 153(100), 179(5)		
Hydroxy-naringenin	8.94	282	287	151(100)	289	153(31), 163(100), 179(23), 271(19)	B-ring	
Naringenin glucuronide isomer 1	7.20	283	447	174(36), 271(100), 429(4)	449	273(100)		
Naringenin glucuronide isomer 2	7.30	289	447	174(21), 271(100), 429(6)	449	273(100), 431(23)		

Naringenin sulfate	7.65	273	351	271(100)	353	ND	
6-Prenylnaringenin (C)	15.21	292, 334	339	219(100), 245(6)	341	285(100)	
Hydroxy-6-prenylnaringenin isomer 1	11.30	293, 336	355	235(100), 261(3)	357	237(24), 285(54), 339(100)	A-ring prenyl
Hydroxy-6-prenylnaringenin isomer 2	11.70	297	355	235(100), 261(4)	357	219(100), 237(44), 285(57), 339(38)	A-ring prenyl
Hydroxy-6-prenylnaringenin isomer 3	11.81	296	355	235(100), 261(4)	357	219(100), 237(43), 285(59), 339(44)	A-ring prenyl
Hydroxy-6-prenylnaringenin isomer 4	12.33	295	355	235(100), 261(4), 283(15), 337(29)	357	219(50), 237(77), 285(86), 301(48), 339(100)	A-ring/ A-ring prenyl
Hydroxy-6-prenylnaringenin isomer 5	14.21	290	355	193(5), 219(100)	357	301(100)	B-ring
6-prenylnaringenin glucuronide isomer 1	11.16	283, 343	515	175(11), 339(100)	517	285(87), 341(59), 461(100)	
6-prenylnaringenin glucuronide isomer 2	12.38	294, 335	515	175(7), 339(100)	517	285(2), 341(10), 461(100)	
Hydroxy-6-prenylnaringenin glucuronide isomer 1	8.75	289, 342	531	175(10), 355(100)	533	339(89), 357(100), 515(68)	A-ring prenyl
Hydroxy-6-prenylnaringenin glucuronide isomer 2	8.88	290	531	175(8), 355(100)	533	339(63), 357(100), 515(72)	A-ring/ A-ring prenyl
Hydroxy-6-prenylnaringenin glucuronide isomer 3	9.10	287	531	175(1), 355(100)	533	301(42), 357(50), 477(100)	B-ring
Hydroxy-6-prenylnaringenin glucuronide isomer 4	10.39	285	531	175(4), 355(100)	533	301(44), 357(46), 477(100)	B-ring
6-prenylnaringenin sulfate isomer 1	11.06	280	419	339(100)	421	ND	
6-prenylnaringenin sulfate isomer 2	11.99	280	419	339(100)	421	ND	
Glabrol (C+C)	16.14	282	391	159(5), 187(25), 203(100)	393	203(8), 205(11), 337(100)	
Hydroxy-glabrol isomer 1	12.55	276	407	203(100), 389(29)	409	205(6), 289(36), 353(18), 391(100),	B-ring/ B-ring prenyl
Hydroxy-glabrol isomer 2	12.77	283	407	203(100), 389(8)	409	205(6), 289(9), 353(12), 391(100)	B-ring/ B-ring prenyl
Hydroxy-glabrol isomer 3	13.15	271	407	187(16), 219(100)	409	203(62), 289(7), 391(100)	A-ring prenyl
Hydroxy-glabrol isomer 4	13.91	282	407	203(100), 349(20), 389(64)	409	205(100), 231(64), 353(93), 391(22)	B-ring/ B-ring prenyl

Hydroxy-glabrol isomer 5	14.26	277	407	203(100), 365(62), 389(18)	409	205(27), 295(49), 353(100), 391(23)	B-ring	
Hydroxy-glabrol isomer 6	14.54	277	407	203(83), 365(100), 389(20)	409	205(15), 295(14), 353(100), 391(31)	B-ring	
Hydroxy-glabrol isomer 7	15.57	282	407	203(100), 219(6)	409	205(49), 231(96), 353(100), 391(10)	UNK	
Dihydroxy-glabrol isomer 1	11.06	291	423	203(23), 219(24), 393(61), 405(100)	425	203(23), 221(3), 389(6), 407(100)	A-ring prenyl	B-ring
Dihydroxy-glabrol isomer 2	11.72	290	423	203(98), 219(30), 393(41), 405(100)	425	205(67), 295(59), 369(100), 407(22)	A-ring	B-ring
Dihydroxy-glabrol isomer 3	11.98	298	423	203(100), 219(30), 393(49), 405(86)	425	205(32), 295(82), 369(100), 407(10)	A-ring prenyl	B-ring
Glabrol glucuronide isomer 1	11.93	284	567	175(12), 391(100), 549(15)	569	393(100), 551(4)		
Glabrol glucuronide isomer 2	12.25	276	567	175(7), 391(100), 549(0.7)	569	393(100), 551(1)		
Hydroxy-glabrol glucuronide isomer 1	9.26	277	583	407(100)	585	ND	B-ring/ B-ring prenyl	
Hydroxy-glabrol glucuronide isomer 2	9.70	283	583	407(100),565(12)	585	ND	UNK	
Hydroxy-glabrol glucuronide isomer 3	10.57	277	583	407(100)	585	ND	UNK	
Glabrol sulfate	12.09	275	471	391(100)	473	ND		

[a] Double bond present in prenyl becomes single bond.
[b] The $[M+H-H_2O]^+$ dominated in the MS^1 compared to the $[M+H]^+$.
DB next to double bond.
ND not determined.
UNK unknown.

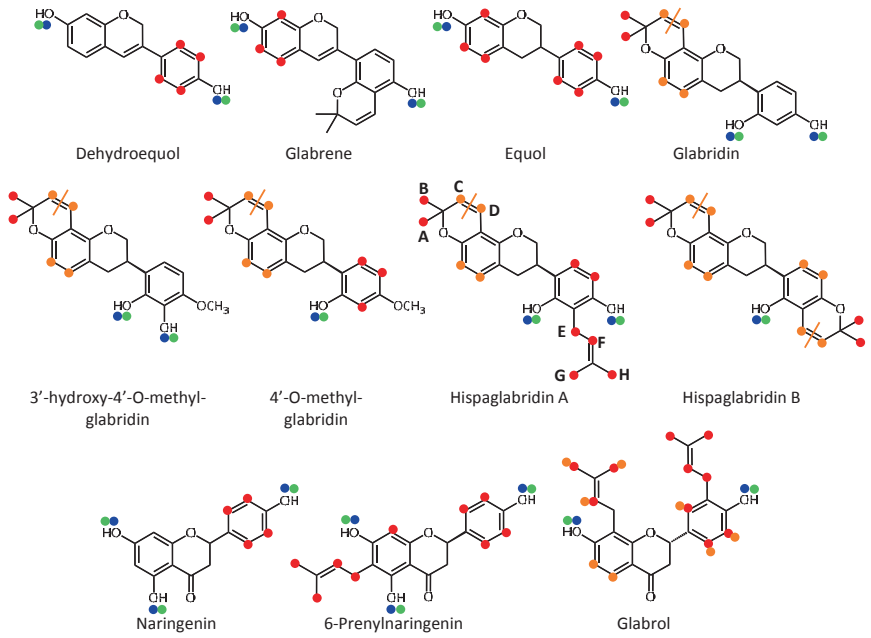
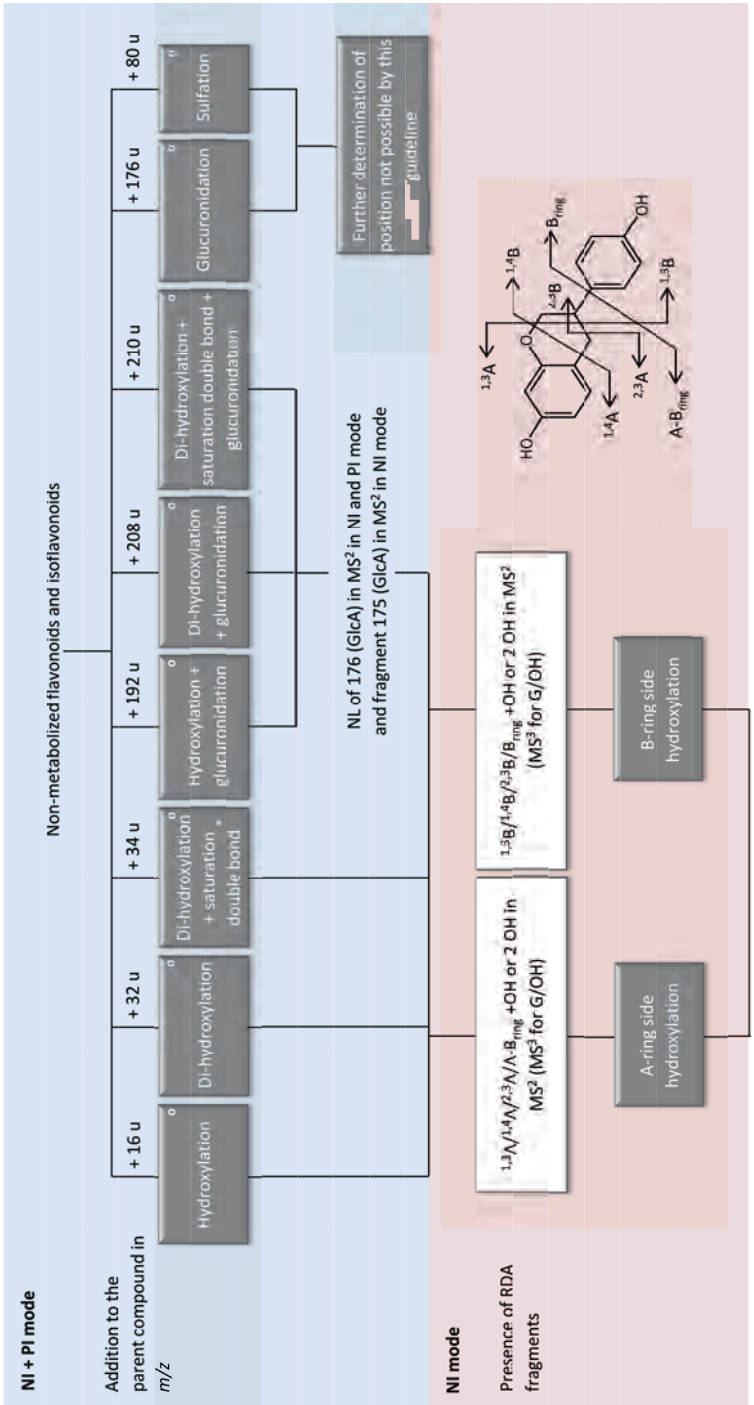


Figure 5.2. Structures of the different flavonoids and isoflavonoids with the possible positions of hydroxyl (●), hydroxyls in case of di-hydroxylation (●) -- = saturation of the double bond in case of di-hydroxylation, glucuronide (●) and sulfate (●) groups. The different positions of substitution to a pyran or chain prenyl are indicated on hispaglabridin A.

Phase I metabolites of flavonoids and isoflavonoids

Mono-hydroxy metabolites. Annotation of the metabolites started with the determination of their *m/z* value in MS^1 in both NI and PI mode. An additional 16 Da compared to the non-metabolized compound, indicated the addition of a hydroxyl group. The interpretation guideline for annotation of metabolites is summarized in Figure 5.3, where this first step is indicated in blue. Further annotation was based on the *retro*-Diels-Alder (RDA) fragments formed in NI mode, as known from previous research[3] (Figure 5.3, indicated in red). Hydroxyl groups might be attached to the A-ring side (aromatic A-ring or A-ring prenyl) or to the B-ring side (aromatic B-ring or B-ring prenyl). Addition of a hydroxyl group to the A-ring, with or without prenyl substituent attached, of the compound was shown to result in RDA-fragments ^{1,3}A, ^{1,4}A, ^{2,3}A, A-B_{ring} in NI MS^2 mode with an additional 16 Da compared to those of the precursor compound. Similarly, hydroxylation at the B-ring resulted in RDA-fragments ^{1,3}B, ^{1,4}B, ^{2,3}B, B_{ring} NI MS^2 mode with an additional 16 Da. For confirmation, RDA-fragments in PI mode MS^2 were analyzed.


NI + PI mode
Non-metabolized flavonoids and isoflavonoids
Addition to the parent compound in m/z
+ 16 u
+ 32 u
+ 34 u
+ 192 u
+ 208 u
+ 210 u
+ 176 u
+ 80 u
Hydroxylation
Di-hydroxylation
Di-hydroxylation + saturation * double bond
Hydroxylation + glucuronidation
Di-hydroxylation + glucuronidation
Di-hydroxylation + saturation double bond + glucuronidation
Glucuronidation
Sulfation
NL of 176 (GlcA) in MS² in NI and PI mode and fragment 175 (GlcA) in MS² in NI mode
Further determination of position not possible by this guideline
NI mode
Presence of RDA fragments
1,3A/1,4A/2,3A/A-B_ring +OH or 2 OH in MS² (MS³ for G/OH)
1,3B/1,4B/2,3B/B_ring +OH or 2 OH in MS² (MS³ for G/OH)
A-ring side hydroxylation
B-ring side hydroxylation
1,3A
1,4B
2,3B
B_ring
OH
HO
1,4A
2,3A
1,3B
A-B_ring

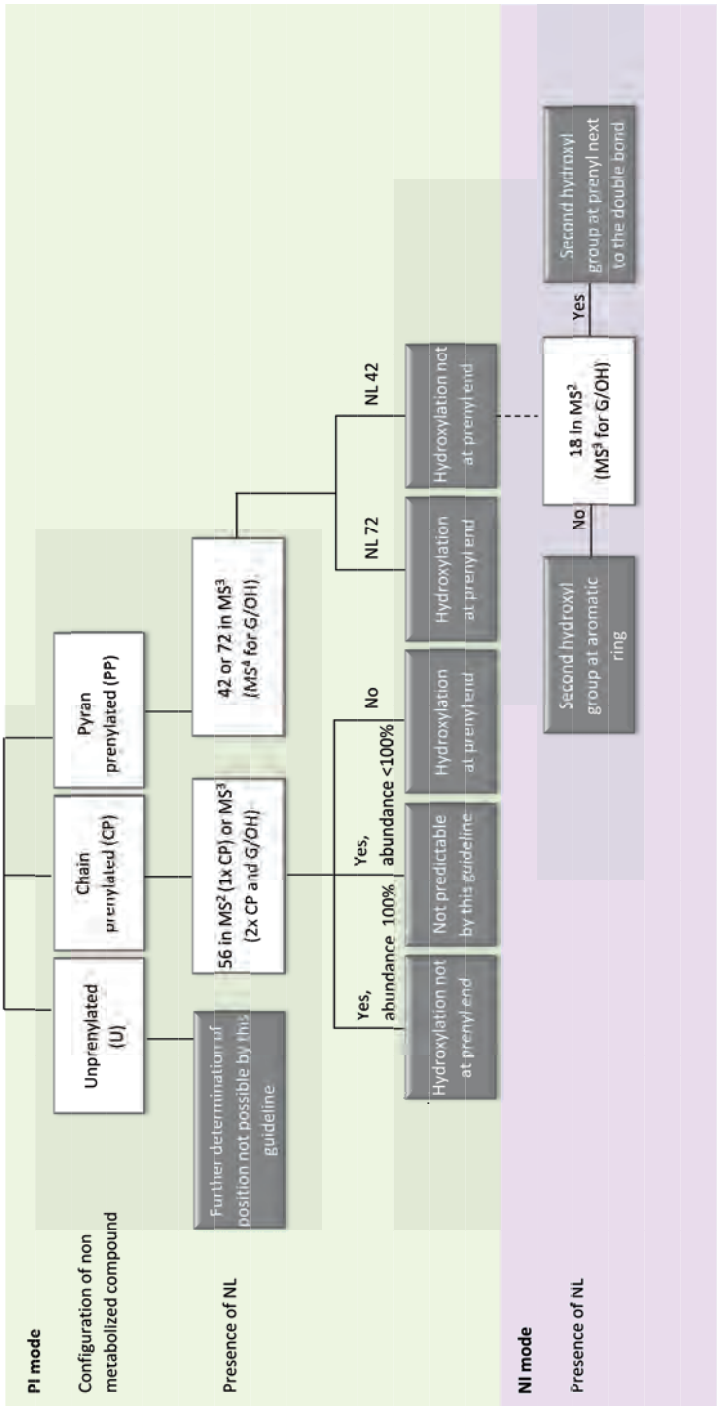


Figure 5.3. Interpretation guideline for determining the structure of phase I and II modified flavonoids and isoflavonoids by MS. ° Metabolite should be compared with the non-metabolized compound. * First hydroxyl group at prenyl next to the double bond (position C or D). Grey boxes indicate decisions; White boxes indicate requirements; Observations are not boxed. Blue = determination of derivative; red = determination of the side of attachment; green = determination of hydroxyl attachment in more detail; purple = determination of the second hydroxyl group in case of dihydroxylation + saturation of the double bond. The dotted line is only possible in case of di-hydroxylation with the loss of the double bond in the pyran prenyl. NL = neutral loss. CP = chain prenylation. G/OH= compounds which are glucuronidated and hydroxylated.

Previous studies showed that specific neutral losses in PI mode MS^2 and MS^3 can predict the type of prenylation[27]. In the current study, these fragmentation patterns were used to analyze whether the hydroxyl group was attached to the prenyl substituent, or directly to the flavonoid or isoflavonoid backbone (Figure 5.3, indicated in green). Compounds with a pyran prenyl have a predominant neutral loss of 42 (representing $-C_3H_6$) in PI mode MS^3. In case of glabridin, a neutral loss of 42 Da was observed in PI mode MS^3 after fragmentation of the RDA-fragment $^{1,3}A$ (*m/z* 205 in PI mode), indicating that the fragmentation pattern was not influenced by hydroxylation. This suggested that the hydroxyl group was not attached to the end of the prenyl (Figure 5.4A). The hydroxyl group might be attached to the aromatic ring or to the small part of the pyran prenyl (positions C or D in Figure 5.2), which is left after fragmentation.

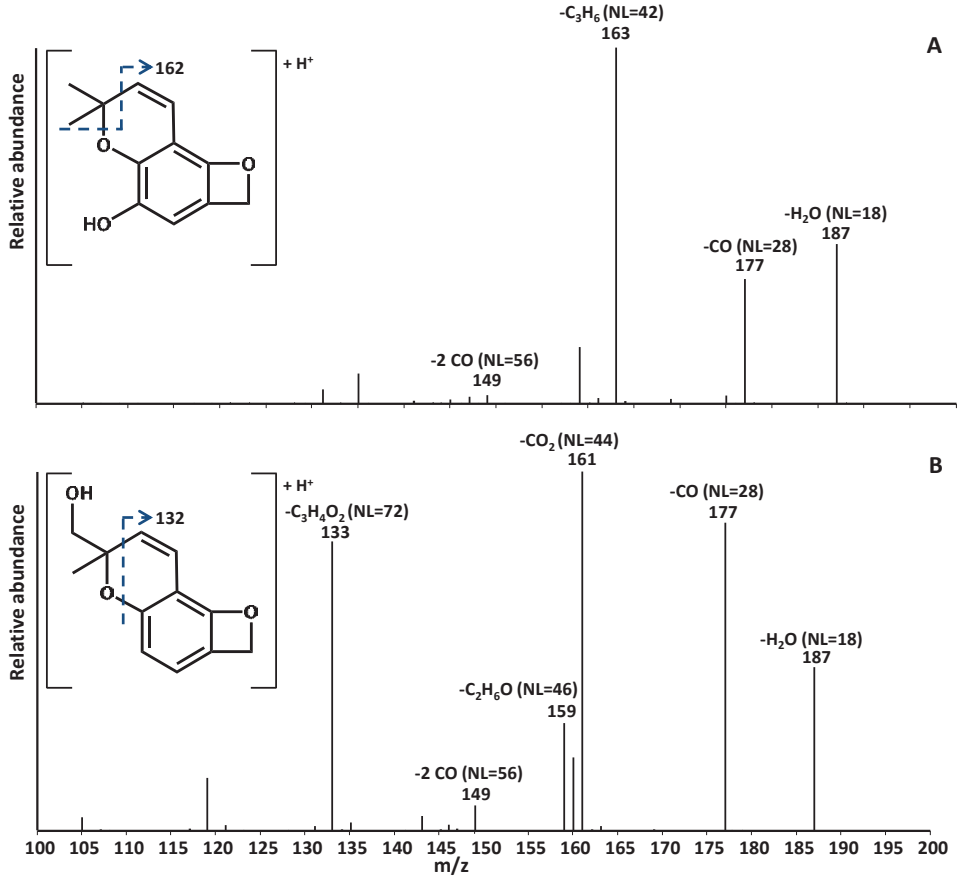


Figure 5.4. MS^3 in PI mode of fragment ion *m/z* 205 of glabridin with the hydroxyl group directly attached to the A-ring (A) or attached to the prenyl end (B). NL = neutral loss.

In other cases, a neutral loss of 72 Da (representing -$C_3H_4O_2$) was observed in PI mode MS^3, instead of the neutral loss of 42 Da (Figure 5.4B). The fragmentation pattern was clearly influenced by hydroxylation, suggesting that hydroxylation occurred at the prenyl end (position A or B in Figure 5.2). Likewise, compounds with a prenyl chain, for example hispaglabridin A, showed a predominant loss of 56 Da (representing –C_4H_8) in PI mode MS.[27] Metabolites with a fragment corresponding to a neutral loss of 56 Da (relative abundance 100%) suggested that the hydroxyl group is not attached to the prenyl end (position F, G or H in Figure 5.2), but to the aromatic ring or to the first carbon of the chain prenyl (position E in Figure 5.2), which is left after fragmentation (Figure S5.1A). The neutral loss of 56 Da likely disappears when the hydroxyl is attached further in the prenyl chain (position G, H and F in Figure 5.2) (Figure S5.1B). For some metabolites the relative abundance of the fragment corresponding to a neutral loss of 56 Da decreased, but did not disappear. In this situation the position of the hydroxyl group could not be predicted. In case of chain prenylation on the A- and the B-ring (glabrol), MS^3 in PI mode of an RDA fragment should be used for the determination of the position of the hydroxyl group, as neutral losses in MS^2 could result from both the A-side prenyl chain and the B-side prenyl chain.

Di-hydroxy metabolites. Double hydroxylation (32 or 34 Da extra, depending on concomitant saturation of the double bond of the prenyl substituent) was observed with prenylated, but not with unprenylated compounds. Double prenylation with saturation of the double bond has also been observed for prenylated pterocarpans in rats[21]. The same strategy as with mono-hydroxylation was used to determine the position of the hydroxyl groups (Figure 5.3). An increase of 34 Da instead of 32 Da was found with pyran prenylated compounds. Interestingly, this 34 Da increase was not observed with chain prenylated compounds. An example of an increase of 34 Da is the pyran prenylated glabridin. Metabolism of glabridin resulted in two different metabolite isomers with an increase of 34 Da. The saturation of the double bond indicated that at least one hydroxyl group was attached to the prenyl pyran (position C or D in Figure 5.2). The second hydroxyl group might be attached to the prenyl pyran or directly to the A-ring. Further determination of the position was performed with PI mode MS^3 (Figure 5.3; indicated in green). A neutral loss of 42 in PI mode MS^3 indicated that the hydroxyl group was attached to the aromatic ring or to the remainder of the prenyl (position C or D in Figure 5.2) remaining after fragmentation. To distinguish between these two positions, NI mode MS^2 was used (Figure 5.3; indicated in purple), as the two isomers showed distinct MS^2 fragmentation in NI mode (Figure 5.5). For isomer 1, different RDA fragments were visible in NI mode MS^2 (Figure 5.5A). This indicated that the second hydroxyl group was attached to the A-ring, as ionization in NI mode most likely occurs on a hydroxyl group attached to the

aromatic ring. This facilitates stabilization of the charge by resonance.[28] Most likely, this will result in RDA fragments. For isomer 2, a dominant loss of water was observed (Figure 5.5B), which indicated that in this case the second hydroxyl group is also attached to the prenyl pyran (positions C or D in Figure 5.2). The elimination of a water molecule is likely enhanced by the vicinal position of the two hydroxyl groups. This probably leads to the formation of an enol moiety, which is subsequently stabilized by keto-enol tautomerism. Interestingly, hydroxylation occurred at the end of the prenyl pyran in case of mono-hydroxylation, whereas the hydroxyl groups were not attached to the prenyl end in case of di-hydroxylation. As hispaglabridin B has RDA fragments corresponding to the A-side (*e.g.* ^{1,3}A), that have the same mass as those corresponding to the B-side (*e.g.* ^{2,3}B), the positions of the hydroxyl groups could not be determined. It is expected that the positions are similar to those of the other pyran prenylated compounds.

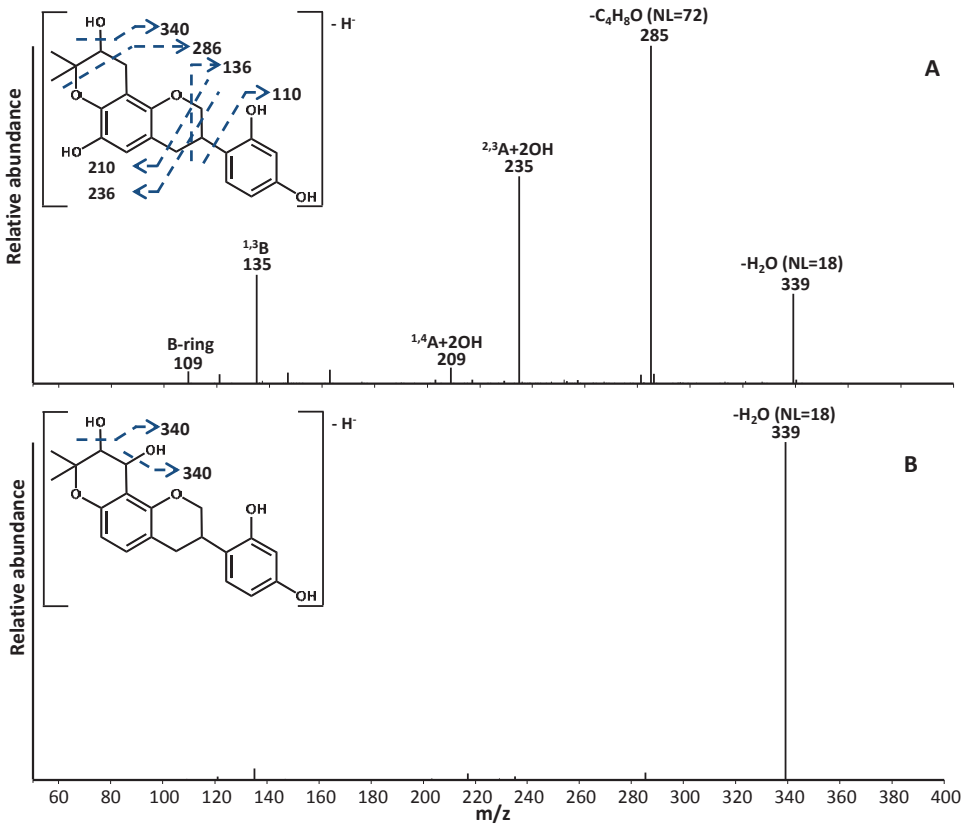


Figure 5.5. MS^2 in NI mode of ion *m/z* 357 of glabridin with the hydroxyl group directly attached to the A-ring and the prenyl pyran (A) or both hydroxyl groups attached to the prenyl pyran (B). NL = neutral loss.

Phase I and II metabolites of flavonoids and isoflavonoids

Glucuronidation. Compounds conjugated with glucuronic acid showed an increase of 176 Da compared to the parent compound (Table 5.1 + Figure 5.3). Different isomers were observed. However, the position of the glucuronic acid could not be determined with UHPLC-ESI-MS, as RDA fragmentation in MS^2 in both NI and PI mode never preceded fragmentation of the glycosidic bond. The fragment (*m/z* 176) corresponding to glucuronic acid was also observed in MS^2 NI mode. It is known that UDP-glucuronosyl transferases have preferred positions for glucuronidation, depending on the isoform of the enzyme[29]. As it is unknown which isoforms are present in the S9 enzyme mix, no indications for the exact positions can be made.

Hydroxylation and glucuronidation. Indeed the combination of phase I and II metabolism led to compounds, which were both hydroxylated and glucuronidated. Fragmentation of these compounds resulted in a dominant fragment of glucuronic acid (*m/z* 175) in NI mode MS^2 and a neutral loss of glucuronic acid in MS^2 in both NI and PI mode. MS^3 in NI and PI mode and MS^4 in PI mode were used to determine the position of the hydroxyl group in a similar way as described above (Figure 5.3). Combination of LC and MS data showed that different isomers were formed after metabolism.

Sulfation. Sulfation of the parent compound resulted in an increase of 80 Da (Figure 5.3). For almost all compounds, only a single metabolite was observed, except for glabridin and 6-prenyl naringenin, where two isomers were formed upon sulfation. No sulfate metabolites were detected for hispaglabridin A. For all compounds the position of the sulfate group could not be determined with UHPLC-ESI-MS, as RDA fragmentation in MS^2 in both NI and PI mode never preceded fragmentation of the bond connection sulfate. Preferential sulfation of isoflavones at the 7-OH position has been shown in human liver cytosol[30]. One might speculate that sulfation occurred at this position, at least when the *C*-7 position is hydroxylated.

Number of isomers and yield of metabolites

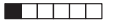
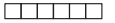
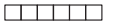
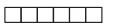
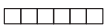
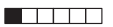
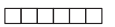
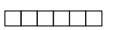
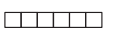
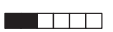
The average number of isomers and the yield, based on the decrease in absorbance of the parent compound (see Materials and Methods), for different subgroups were determined for phase I, phase I + II and phase II sulfation only (Table 5.2). Compounds were classified in subgroups in different ways based on three criteria: backbone structure (subgroups: isoflavene, isoflavan or flavanone), number of prenyl groups (subgroups: 0, 1 or 2), and prenyl configuration (subgroups: chain or pyran). Overall, it was observed that the yield of glucuronidation was dominant over the yield of hydroxylation (Table 5.3).

Table 5.2. Proportions of the parent compound (%) and the number of isomers for the different metabolism phases per compound.

Identification	Parent compound (%)[a]	Number of isomers
Dehydroequol	100	
Phase I	79 ± 4	1
Phase I + II	1 ± 1	2
Phase sulfate	90 ± 9	1
Glabrene	100	
Phase I	61 ± 6	1
Phase I + II	1 ± 0	2
Phase sulfate	74 ± 18	1
Equol	100	
Phase I	67 ± 2	2
Phase I + II	1 ± 0	2
Phase sulfate	94 ± 2	1
Glabridin	100	
Phase I	80 ± 11	4
Phase I + II	16 ± 1	5
Phase sulfate	86 ± 0	2
3'-Hydroxy-4'-*O*-methyl-glabridin	100	
Phase I	62 ± 1	4
Phase I + II	12 ± 0	2
Phase sulfate	83 ± 5	1
4'-*O*-Methyl-glabridin	100	
Phase I	99 ± 1	5
Phase I + II	74 ± 1	4
Phase sulfate	99 ± 0	1
Hispaglabridin A	100	
Phase I	97 ± 1	9
Phase I + II	44 ± 12	8
Phase sulfate	100 ± 0	0
Hispaglabridin B	100	
Phase I	67 ± 13	4
Phase I + II	24 ± 5	4
Phase sulfate	71 ± 0	1
Naringenin	100	
Phase I	74 ± 10	1
Phase I + II	1 ± 1	2
Phase sulfate	80 ± 3	1
6-Prenylnaringenin	100	
Phase I	95 ± 3	5
Phase I + II	18 ± 2	6
Phase sulfate	95 ± 5	2
Glabrol	100	
Phase I	30 ± 4	10
Phase I + II	11 ± 4	5
Phase sulfate	96 ± 0	1

[a] Percentages were determined as follows: $\frac{\text{peak area at 280 nm of the parent compound after incubation}}{\text{peak area at 280 nm of the parent compound at t=0}}$ x 100%.

Table 5.3. Average number of isomers and average yield of metabolites for different subclasses of (iso)flavonoids, number of prenyl groups and prenyl configurations.

	Phase I (hydroxylation)		Phase I + II (glucuronidation)		Phase sulfate (sulfation)	
	# isomers	yield[b]	# isomers	yield[b]	# isomers	yield[b]
Backbone structure						
Isoflavene (2[a])						
Isoflavan (6)						
Flavanone (3)						
Number of prenyl groups						
Unprenylated (3)						
Single prenylated (5)						
Double prenylated (3)						
Prenyl configuration						
Prenyl pyran (5)						
Prenyl chain (2)						

[a] Number of representatives. Hispaglabridin A is not taken into account for the subgroup prenyl configurations, as it had one chain and one pyran prenyl group.

[b] Yield = 100% - percentage parent compound remaining. Percentage parent compound = $\frac{\text{peak area at 280 nm of the parent compound after incubation}}{\text{peak area at 280 nm of the parent compound at t=0}}$ x 100%.

It should be noted that the average yield of the different metabolites is based on the decrease of the parent compound, and not on the sum of metabolites (Table 5.2), as the molecular extinction coefficients of the metabolites formed were unknown. The yield of hydroxylation was between 20 and 50% and the yield of sulfation was <20% for all subgroups. The yield of glucuronidation varied from 70% for isoflavans to 100% for isoflavenes. This *in vitro* outcome is in line with *in vivo* findings, as it is known that glucuronidation is the most dominant during phase II metabolism of drugs in human.[31] Moreover, glucuronide metabolites are also more abundant than sulfate metabolites when the isoflavonoids daidzein and genistein are metabolized.[32,33] The number of hydroxyl and glucuronyl isomers varied between 1 and 10 for the different subgroups, whereas the number of sulfate metabolite isomers was not influenced to a big extent by the different subgroups. For most compounds only one sulfate metabolite was formed. The low number of sulfate isomers could be due to the fact that phase II sulfation was not combined with phase I metabolism, which might be needed for efficient sulfation. Concentrations of sulfate metabolites below the detection limit of the MS might be another explanation.

DISCUSSION

In vitro metabolism of 11 different prenylated (iso)flavonoids, using pork liver preparations, resulted in the formation of 100 different metabolites, *i.e.* hydroxyl, glucuronyl, both hydroxyl and glucuronyl, and sulfate metabolites (Table 5.1). Lower yields of sulfate (<20%) and hydroxyl (20-50%) metabolites were formed compared to glucuronyl metabolites (70-100%). Nevertheless, these metabolites could be studied in detail due to the separation of the different metabolic phases.

Opportunities and limitations for using the interpretation guideline

An interpretation guideline for the determination of the position of the attachment of the hydroxyl, glucuronic acid and sulfate groups was made and is illustrated in Figure 5.3. The position of glucuronidation and sulfation could not be determined by UHPLC-MS^n, as these groups will be fragmented first in MS^2. However, for hydroxylation a more in-depth characterization of various hydroxyl isomers was possible by MS. Only for one metabolite, which had two hydroxyl groups on the same side of the molecule (A-side or B-side), this guideline was not conclusive. Still, an indication of the side of hydroxylation could be made, but an exact position could not be determined. This interpretation guideline might be extrapolated to study metabolism of extracts in which the compounds present are known. However, one limitation is observed in this case. If two non-metabolized compounds in the extracts differ in one hydroxyl group,

for example 4'-*O*-methyl-glabridin and 3'-hydroxy-4'-*O*-methyl-glabridin, it is not possible to distinguish between the hydroxyl metabolite of 4'-*O*-methyl-glabridin and the non-metabolized 3'-hydroxy-4'-*O*-methyl-glabridin, as MS^n spectra will be similar. Theoretically, it is also possible that a non-metabolized compound and a metabolized compound have the same mass. For example, the non-metabolized glabrene has the same mass as the metabolite equol sulfate. In this case a distinction can still be made in MS^2 (Figure 5.3, indicated in red) as the compounds have distinct MS^2 fragmentation patterns. So, the first step with MS^1 (Figure 5.3, indicated in blue) is not conclusive on the derivatization in this situation.

Structure-metabolism relationships

Clear differences between the different backbone structures were observed with respect to the average number of hydroxyl metabolites formed. Only one isomer was found for the isoflavene subclass, whereas an average of 5-6 isomers were found for the isoflavan and flavanone subclasses (Table 5.3). Also with glucuronidation, isoflavenes had the lowest average number of isomers. With respect to yield, the extent of glucuronidation of isoflavenes was higher than that for isoflavans and flavanones. This was not the case for hydroxylation. The differences observed in metabolic routes of different backbone structures might find their origin in differences in planarity: isoflavenes have an extra double bond in the C-ring, which makes this subclass more planar than the isoflavans and flavanones.

Different kinds and number of prenyl groups also affected metabolism. With increasing number of prenyl groups, it was observed that more hydroxyl isomers were formed without compromising the total yield of hydroxylated products, especially when a prenyl chain is present (Table 5.3). The flexibility of the prenyl chain might facilitate various positions of hydroxylation by cytochrome P450 enzymes. Consistent with hydroxylation, more glucuronyl isomers were found with increasing number of prenyl groups, in particular prenyl chains. As expected, glucuronidation is facilitated at more positions when molecules are first hydroxylated during phase I metabolism. Interestingly, the yield of glucuronyl metabolites was higher for unprenylated compounds (90-100%) than that for single and double prenylated compound (70-80%) (Table 5.3). Possibly, prenyl groups hinder the attachment of glucuronic acid by UDP-glucuronosyltransferases. No large differences in yield of glucuronidation were observed between the different kinds of prenyl configuration.

When the above would also be valid in an *in vivo* situation, it can be postulated that prenylation of flavonoids and isoflavonoids might have interesting consequences. As prenylation negatively affects the extent of glucuronidation,

prenylated metabolites might be excreted slower from the human body, as hydroxylation only might not increase polarity sufficiently enough for efficient clearance.[18] With reduced extent of glucuronidation, a larger proportion of the parent compound and its hydroxyl metabolites will reside in the unconjugated form, which might enhance the overall estrogenic activity, as some of the hydroxyl metabolites might still be estrogenic or even more estrogenic than the parent compound.[11,12] It was shown before that hydroxylation might result in more active metabolites.[9,10] For example, 4-hydroxy-tamoxifen has a 100-fold higher affinity for hERs, and is more potent in suppressing cell proliferation compared to the non-metabolized tamoxifen.[9,10]

In conclusion, the extensive hydroxylation of prenylated molecules, together with their reduced glucuronidation and consequently delayed clearance, increases the half-life of hydroxyl metabolites inside the human body. Hence, they might still have estrogenic activity. Therefore, determination of the estrogenic activity of hydroxylated metabolites of prenylated (iso)flavonoids is of importance.

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SUPPORTING INFORMATION

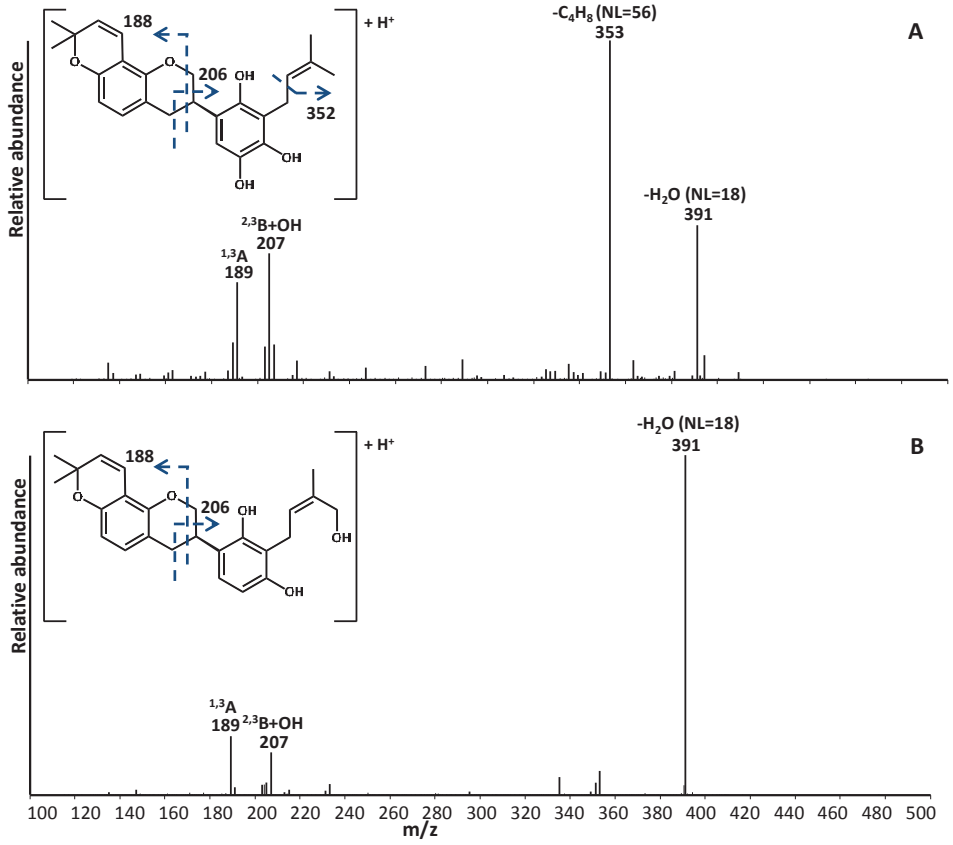

Figure S5.1. MS^2 in PI mode of ion *m/z* 409 of hispaglabridin A with the hydroxyl group attached to the B-ring (A) or attached to the prenyl chain (B). NL = neutral loss.

Chapter 6

General Discussion

As described in the **General Introduction** of this thesis, isoflavonoids and flavonoids can bind to hERs. Upon consumption this might result in beneficial or adverse health effects *in vivo*. Prior to our research, it was shown that prenylation can modulate estrogenic activity and that structure-activity relationships needed to be elaborated. In this thesis research prenylated flavonoids and isoflavonoids were purified and their *in-vitro* estrogenic properties were determined (**Chapters 3** and **4**). One of the main findings was that the different modes of action, caused by different kinds of prenylation, can be related to structural features of the estrogen receptor (**Chapter 3**). Another important outcome of our research was that prenylation is a key factor in determining SERM behavior, whereas additionally the backbone structure is important for determining subtype-selectivity (**Chapter 4**). It was also shown that heat and acid affected the stability of 6a-hydroxy-pterocarpans and consequently their estrogenicity (**Chapter 2**). Last, a key finding was that more hydroxyl isomers and a lower yield of glucuronidation was observed for prenylated (iso)flavonoids than for unprenylated (iso)flavonoids during phase I and II metabolism (**Chapter 5**). The current chapter discusses the findings mentioned above by addressing structural characteristics that influence estrogenicity and comparing compounds with promising estrogenic behavior, found by us, with known estrogens from the literature. In addition, other aspects, like the activity towards other receptors, the methods used and prospects for the use of prenylated (iso)flavonoids are discussed.

STRUCTURAL CHARACTERISTICS DEFINING ESTROGENICITY OF NATURAL COMPOUNDS

Table 6.1 shows a summarized comparison of the modes of estrogenic action of flavonoids and isoflavonoids studied in this thesis (**Chapters 3** and **4**) with estrogenic compounds already described in literature and analyzed by different methods (**Chapter 1**). With respect to the yeast bioassays, only results for hERα are taken into account in the comparison, as it was shown that prenylation especially modulated the response towards this receptor (**Chapter 4**). The numbers in the table indicate the number of compounds that have been tested in a particular bioassay; the actual number of compounds is less as there is overlap in molecules tested in these bioassays. With the compounds purified in this thesis research, the set of prenylated (iso)flavonoids with known estrogenic activity was extended. The new compounds described matched well with the emerging view from the literature (Table 1.2; **Chapter 1**). The statement that prenylation modulates the mode of estrogenic action is more strongly established with our research than was before.

Table 6.1. Overview of the mode of estrogenic action of unprenylated and prenylated (iso)flavonoids towards hERα in different bioassays. Numbers indicate the number of compounds tested.

Prenylation (position)	Thesis	Literature						
	hERα Yeast	hERα Yeast	MCF-7	T47D	Ishi.	HEK-293	UMR-106	U2OS
None	3	11 1	17 3	5	7	3		
Chain	4 1 1	1 1	7		4	1	1	1
Pyran (δ)	1	1	2	1		1	1	1
Pyran/Furan (α or β)	2 7 1	1	7 4	4		1		
Double chain	1	7	3				1	1
Chain + pyran/furan	1	5	2					

Green = agonist; red = antagonist; white = no response; grey = not determined. Ishi. = Ishikawa cells.

In general, our data (Table 6.1, **Chapters 3** and **4**) show that unprenylated, chain and δ-position pyran prenylated (iso)flavonoids show an agonistic mode of action towards hERα in the yeast bioassay. This is in line with previous outcomes using different bioassays (Table 1).[1-20] Deviations were observed, as some compounds showed an antagonistic mode of action (Table 6.1).[16 19] Nevertheless, 73 of the 79 unprenylated, chain and δ-position pyran prenylated (iso)flavonoids were agonistic. The prenyl chain is supposed to fit into a hydrophobic pocket of the ligand binding domain of the receptor, thereby evoking an agonistic mode of action (**Chapter 3**). The agonistic mode of action of compounds with pyran prenylation on the δ-position might find its origin in the fact that the length of these compounds is unchanged (**Chapter 3**). An antagonistic mode of action was mainly observed for compounds that are pyran/furan prenylated on the α or β position (Table 6.1). It is suggested that the antagonistic mode of action resulted from the increase in the length of the compound (**Chapter 3**). However, two of such compounds in our research (glyceollin I and glyceofuran, **Chapter 4**), as well as some compounds described in literature, had an agonistic mode of action (Table 6.1).[20,21] An agonistic mode of action was especially observed in cell lines based on breast cells (MCF-7 and T47D). In literature, the furan prenylated glyceollin III, showed both agonistic and antagonistic mode of action, indicating that this compound can act as SERM.[22,23] Double chain prenylation of flavonoids led to an antagonistic mode of action, which is in line other findings (Table 6.1).[8,10,24] The prenylated isoflavan hispaglabridin A did not show any estrogenic response in our research. However, in literature different isoflavones and one flavanone with chain + pyran/furan prenylation were reported to have an antagonistic mode of action using a yeast bioassay.[8,10] The occurrence of the (few) discrepancies described above indicate that different assays can result in different responses. It also indicates that other characteristics, *e.g.* different backbone structures or different positions of prenylation, are of importance for the modes of estrogenic action.

Elaborating on backbone structure, it was observed that this was mainly of importance for the estrogenicity towards hERβ in the yeast assay (**Chapter 4**). In Table 6.2, an overview of the modes of estrogenic action of different backbone structures with different kinds of prenylation are shown. Unprenylated, chain and δ-position pyran prenylated compounds, with an agonistic mode of action towards the yeast hERα (Table 6.1), showed the same mode of action towards hERβ. For this situation, it can be concluded that the backbone structure is not of big importance. On the other hand, it was observed that the backbone structure is of large importance for compounds with an antagonistic mode of action towards hERα (Tables 6.1 and 6.2). α/β-Position pyran prenylated 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes all have an agonistic mode of action towards hERβ, whereas α/β-position pyran prenylated isoflavans had an antagonistic mode of action towards both hERs (**Chapter 4**). It is speculated that only compounds with an extra D-ring show a different mode of action towards the hERs, whereas compounds without the extra D-ring will show the same mode of action towards both hERs. In line with this, it is expected that α/β-position furan prenylated isoflavans will also give an antagonistic mode of action towards hERβ, whereas α/β-position furan prenylated 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes have an agonistic mode of action towards hERβ.

Table 6.2. Overview of the mode of estrogenic action of unprenylated and single prenylated (iso)flavonoids towards hERβ. Numbers indicate the number of compounds tested.

Backbone	**hERβ Yeast**				
	Un-prenylated	**Chain prenylated**	**Furan prenylated (α/β)**	**Pyran prenylated (δ)**	**Pyran prenylated (α/β)**
6a-Hydroxy-pterocarpan		2	3		2
Flavone	1	2			
Isoflavan	1				2
Isoflavene	1			1	
6a,11a-Pterocarpene		1	1		2

Green = agonist; red = antagonist; white = no response; grey = not determined.

In this thesis six SERMs with an agonistic mode of action towards the hERα in the CALUX bioassay and an antagonistic mode of action in the hERα yeast bioassay were identified (**Chapter 4**). An antagonistic mode of action towards the hERα in the yeast bioassay is probably caused by the increased length due to pyran or furan prenylation (**Chapters 3** and **4**). It is unclear why the compounds with an increased length still have an agonistic mode of action towards the hERα in the CALUX bioassay. Probably this is caused by different cellular environments, *e.g.* differences in cofactors/corepressors. Also other SERMs, like 8-prenylgenistein (8DMAG) and the

unprenylated luteolin, displayed an antagonistic mode of action in a yeast bioassay, but they did not have an increased length.[8,16,19] It should be emphasized that the opposite is also possible, *i.e.* compounds acting as agonist in yeast cells and as antagonist in other cell types (*e.g.* tamoxifen).

COMPARISON OF THE ACTIVITY OF PRENYLATED (ISO)FLAVONOIDS TO KNOWN ESTROGENIC COMPOUNDS

Given the importance of prenylation of (iso)flavonoids for modulating estrogenicity, we here relate their modes of action and affinities, as determined in this thesis, with those of known (synthetic) estrogenic compounds.

Agonistic mode of action

In Figure 6.1, structures and REP values of the most potent partial agonist, with a mixed agonistic/antagonistic activity in the yeast bioassay (glabrene) and the two most potent full agonists (dehydroequol and 8-prenylnaringenin) from this research, as well as three agonists from literature (including the reference compound E_2), are shown. All compounds have a similar length (~10.5-12 Å, measured with MOE, **Chapters 3** and **4**) between the two most distant hydroxyl groups, similar to E_2, except for WAY-169916 that has a fluorine group instead of a hydroxyl group. Compounds are considered estrogenic when they are able to activate the hER at concentrations below 10^4 times the concentration at which 17β-estradiol activates the receptor.[25] Thus, REP values > $1x\ 10^{-4}$ indicate that compounds have clear estrogenic properties. According to the REP values obtained in the yeast bioassay, dehydroequol and 8-prenylnaringenin are relatively strong estrogenic compounds (Figure 6.1 and **Chapter 3**), with REP values 4 and 11 times the REP value of the well-known natural phytoestrogen genistein, respectively.[15] Glabrene, classified as a partial agonist in this research, has a relatively low REP value (Figure 6.1). It should be stated that direct correlation of REP values to *in vivo* effect is not self-evident, as the synthetic partial agonist WAY-169916 displays low REP values in MCF-7 cells.[26] As WAY-169916 has been shown to induce beneficial effects *in vivo*[27], REP values < $1x10^{-4}$ in *in vitro* assays are not necessarily a disqualifier for a compound.

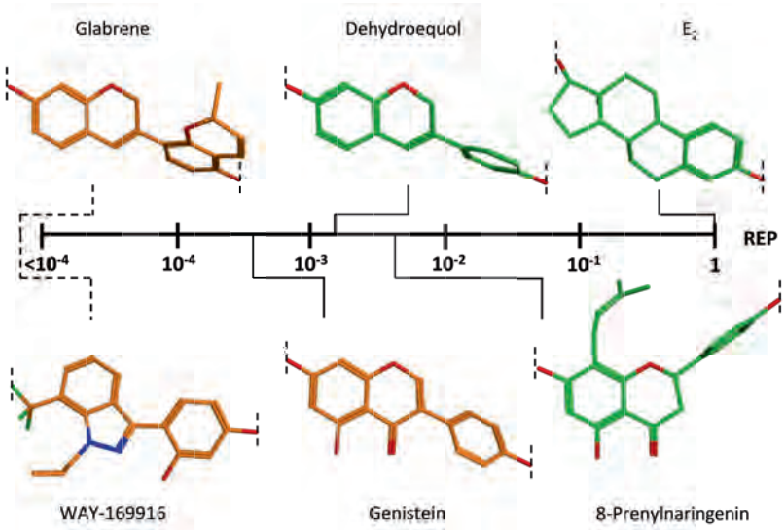

Figure 6.1. Minimalized molecular structures and REP values towards hERα of agonists (green) and partial agonists (orange). Length is measured between the atoms indicated with the dotted lines. Exact REP values could not be determined for glabrene and WAY-169916 as higher concentrations were toxic or water-insoluble.

Antagonistic mode of action

Glabridin and 4-*O*-methyl-glabridin showed an antagonistic mode of action towards both hERs in the yeast bioassays (**Chapter 3**). Figure 6.2 shows that distinct structural differences exist between these antagonists and the known hERα antagonist ICI 182,780. ICI 182,780 has an additional "tail", which is not present in glabridin and 4-*O*-methyl-glabridin. Moreover, the distance between the hydroxyl groups of ICI 182,780 is similar to that of E_2 (~11Å), whereas the length of glabridin and 4-*O*-methyl-glabridin is increased (~12-13.5Å). The differences in structural characteristics explain their different ways of inducing antagonism. The "tail" of ICI 182,780 displaces helix 12 in a direct way and, therefore, it is classified as a direct antagonist. The antagonistic compounds found in this research act as indirect antagonists, as they displace helix 12 indirectly by colliding with helix 11 (**Chapter 3**). At a concentration of 10^{-5} M, glabridin and 4-*O*-methyl-glabridin (**Chapter 3**), gave an antagonistic signal reduction of 90 and 63% in the hERα yeast bioassay, respectively, whereas ICI 182,780 showed a reduction of only 27%.[1] This suggests that glabridin and 4-*O*-methyl-glabridin might be promising as templates for the design of indirect antagonists.

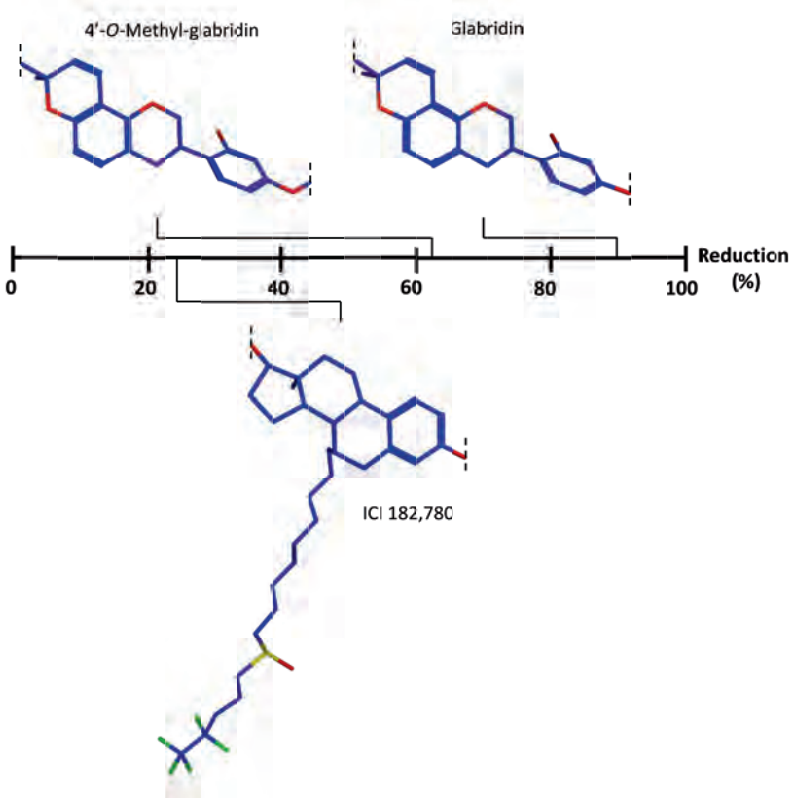

Figure 6.2. Minimalized molecular structures and antagonistic signal reduction towards hERα in the yeast bioassay at a concentration of 10^{-5} M.

SERM behavior

Prior to the start of this PhD research, it was suggested that prenylation might be an important determinant for inducing SERM behavior and the prenylated isoflavonoids glabridin, phaseollin, 8-prenylgenistein and kievitone were classified as such.[28] In this thesis, six additional prenylated isoflavonoids were identified to have SERM behavior (**Chapter 4**). Glyceollin I, II and III were classified as antagonists in most previous studies.[22,29] This mode of estrogenic action was also observed towards hERα in the yeast bioassay in our studies. However, they had an agonistic mode of action in the hERα CALUX bioassay, thereby classifying them as SERMs. Glyceollin V, dehydroglyceollin II and IV were the other three SERMs observed in our research (**Chapter 4**). It is thus expected that more prenylated isoflavonoids, currently classified as either agonist or antagonist, will turn out to be SERMs if the number of studies in which these compounds are tested using different cell assays grows.

All SERMs identified in this research have structural characteristics comparable to glyceollin II (Figure 6.3). Glyceollin II, is one of the most potent SERMs identified in this research. SERMs already used for their beneficial effects, like tamoxifen and raloxifene, have structural characteristics different from glyceollin II (Figure 6.3) and the other SERMs found in our research. These structural differences explain the manner by which they cause antagonism, indirect antagonism via helix 11 vs direct antagonism via helix 12, as described above in more detail. However, the agonistic mode of action of glyceollin II cannot be explained by its structural characteristics. The REP value of glyceollin II was higher than those of tamoxifen and raloxifene, indicating that glyceollin II had a stronger agonistic activity (Figure 6.3).[1] On the other hand, tamoxifen and raloxifene seem to be more potent antagonists, as IC_{50} values are lower.[30] Care should be taken for this comparison as the IC_{50} values were obtained with different assays.

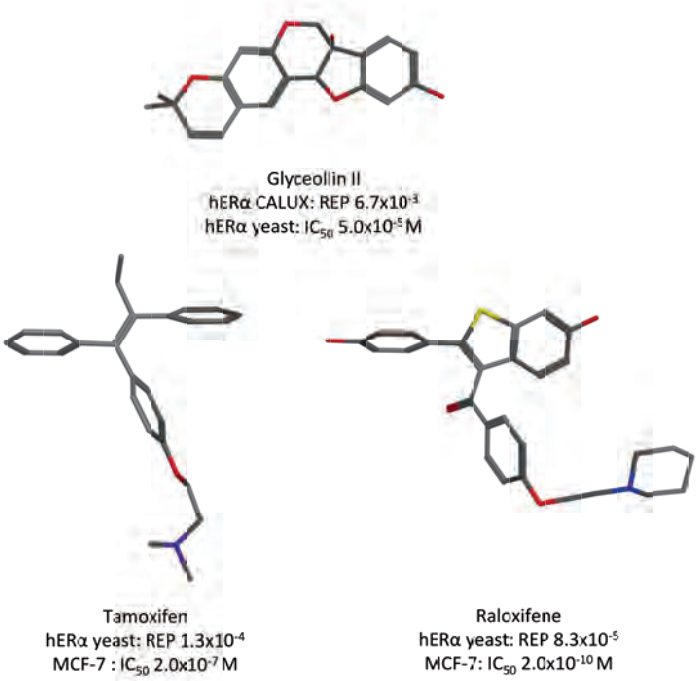

Figure 6.3. Minimalized molecular structures and estrogenic activities of SERMs.

ER subtype-selectivity

In this research, eight ER subtype-selective compounds were identified, *i.e.* compounds with a different mode of action towards hERα than towards hERβ (**Chapter 4**). Two ER subtype-selective compounds identified in our research, glyceollin III and dehydroglyceollin III, are shown in Figure 6.4. All ER subtype-

selective compounds identified in this thesis have comparable structural characteristics. They all belonged to the subclasses of 6a-hydroxy-pterocarpans and 6a,11a-pterocarpenes, which contain an extra D-ring in contrast to *e.g.* isoflavans, isoflavenes and flavanones. The known ER subtype-selective compound *R,R*-THC (Figure 6.4) does not show similar structural characteristics with these new compounds. The fact that pterocarpans/pterocarpenes and *R,R*-THC all show subtype-selectivity, despite structural differences, might be related to the former being hERα antagonists and hERβ agonists, whereas this is the opposite for *R,R*-THC. The ER subtype-selective compounds found in this thesis showed their agonist or antagonist activities only at concentrations ~ $1x10^{-5}$ M, whereas *R,R*-THC displays estrogenic activity already at ~ $1x10^{-7}$ M.[31] Nevertheless, the opposite pattern of mode of action of pterocarpans/pterocarpenes compared to *R,R*-THC might provide opportunities for drug development. Theoretically, patients with ER-positive breast cancer would benefit the most from treatment with molecules exhibiting this rare pattern.[32] This correlates well with the activity pattern for subtype-selective compounds described in this thesis.

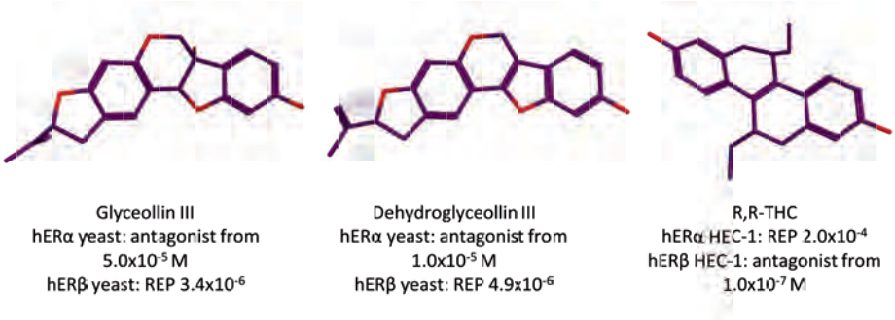

Figure 6.4. Minimalized molecular structures and estrogenic activities of ER subtype-selective compounds with a different mode of action towards hERα and hERβ.

The most striking structural feature of the subtype-selective compounds found in this research is that they all had an extra D-ring compared to other (iso)flavonoids, which is also observed with coumestans. According to the observations described (**Chapter 4**), a prerequisite for coumestans to become an ER subtype-selective compound, would be prenylation (which will cause antagonism towards hERα) of the coumestan backbone. Prenylated coumestrol was the only prenylated coumestan present in the extract of soybean seedlings elicited by fungus, although in low concentrations (**Chapter 2**), similar to previous experiments.[33] Because of its low abundance, prenyl coumestrol was not purified in this thesis. Nevertheless, it is worthwhile to investigate this compound for potential subtype-selective behavior, as coumestrol is known to have high affinity for ER.[15]

DIFFERENT DOCKING POSES OF SERMS

Previously, glyceollin I was proposed to dock in a similar pose in hERα as the direct antagonist tamoxifen[22] (Figure 6.5B) and consequently it was suggested that glyceollin I acts as a direct antagonist. However, that docking pose resulted in tight torsion angles around the *C*-6a and *C*-6 position (Figure 6.5B), which might be less favorable. Applying *in silico* modelling (using MOE, **Chapter 3**), we suggest that all SERMs with only antagonistic activity towards the hERα in the yeast bioassay bind the estrogen binding pocket in a similar way as described before for the prenylated isoflavans[34] (**Chapter 4**; Figure 6.5A). In this way, it is thought that they act as indirect antagonists, with less tight torsion angles. However, the minimized energy of glyceollin I after docking is similar for the direct and indirect poses (~ -548 kJ), indicating that both poses might be possible. It should be noted that glyceollin I was docked in the hERβ crystal structure[35] in the present study instead of hERα, as no hERα crystal structure with the displacement of helix 11 was available. It is assumed that hERβ will show the same behavior as hERα. It is clear that the distinction between direct and indirect antagonists can only be made with help of crystal structures of the hER and cannot be made with the results from estrogenic assays and/or *in silico* modelling only. Nevertheless, it became clear that besides the docking position described in literature[22], another docking pose for SERMs is just as likely.

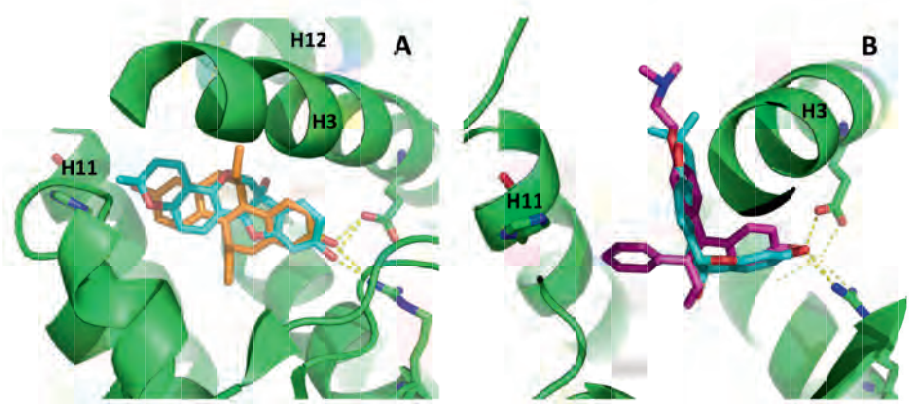

Figure 6.5. Ligand binding cavity in complex with the indirect antagonist *R,R*-THC (orange) in hERβ (A), the ligand binding cavity in complex with the direct antagonist 4-hydroxytamoxifen (purple) in hERα (B) and the two possible docking positions of glyceollin I (blue) according to this research (A) and previous research (B).[22]

DETERMINATION OF SERM AND ER SUBTYPE-SELECTIVE BEHAVIOR OF (ISO)FLAVONOIDS

Determination of the estrogenic activity in this research was performed with hERα and hERβ yeast bioassays and an U2-OS based hERα CALUX bioassay. Mixed agonistic and antagonistic activity in the yeast bioassay shows that a compound behaves as a partial agonist. However, it can also hint at SERM behavior.[36] To confirm the classification of such compounds as a SERM, the compound has to be tested in at least one other bioassay. In Table 6.3, it is shown that SERM activity can correlate with different patterns in mode of action using various cell lines. Different *in vitro* transcriptional activation assays can be used to determine the mode of action of a compound. Most cell lines can contain both hERα and hERβ. As a consequence, SERM behavior can also be caused by differences in abundance of hERα and hERβ, and not only to different cellular environments alone. Moreover, if cell lines with differences in the abundance of the two hERs are used, the individual activity towards one particular hER cannot be determined. For example, uterus cells cannot be used to study ER subtype-selectivity as both receptors are present (Table 6.3). The bioassays used in this research only expressed a single hER. So, the activity observed is the result of that particular receptor. In this way, ER subtype-selectivity and SERM behavior based on differences in cellular environment alone could be determined.

Table 6.3. Mode of estrogenic action of different SERMs in different assays. SERMs are indicated in bold.

	Yeast	**Breast (MCF-7; T47D)**		**Bone (U2-OS; UMR-106)**	**Uterus (NCC16; NCE16)**	**Kidney (HEK-293)**
	hERα or hERβ	Mainly hERα[37]		Mainly hERβ[38]	hERα and hERβ[39]	Mainly hERα[39]
E_2	Ago	Ago		Ago	Ago	Ago
Tamoxifen	Ago[1]	Anta[40]		Ago[41]	Ago[42]	Unk
Raloxifene	Ago[1]	Anta[40]		Ago[43]	N.r.[40]	Unk
Glabridin	Anta	Ago[20]		Unk	Unk	Unk
Glyceollin I	Ago[a]	Ago[b 23]	Anta[b 22]	Ago[c]	Unk	Anta[29]
ICI 182,780	Anta[1]	Anta[44]		Anta[45]	Anta[46]	Anta[47]

Ago = agonist; Anta = antagonist; Unk = unknown; N.r. = no response.

The superscripts refer to the literature references.

[a] Partial agonist/SERM towards hERα, agonist towards hERβ.

[b] Different activity towards MCF-7 cells obtained in different studies.

[c] Estrogenicity determined with a hERα-CALUX bioassay, which are transgenic U2-OS cells containing only hERα and no basal level of other nuclear receptors.

Classification of estrogenic compounds might be ambiguous. In the literature, ER subtype-selectivity is associated either with differences in affinity (EC_{50}, REP) towards the two hERs or with different modes of action towards the two hERs (**Chapter 1**). In our opinion, ER subtype-selectivity should refer to the mode of action (*e.g.* agonist, antagonist) rather than to affinity for only one receptor. The variation in affinities reported in the literature for agonists, classified as subtype-selective, is large, ranging from 10 to 450-fold[15,48], which makes classification as ER subtype-selectivity rather arbitrary. Therefore, we prefer to reserve the annotation of subtype-selectivity for molecules with clearly opposite mode of action.

CAN PRENYLATION MODULATE THE ACTIVITY TOWARDS RECEPTORS OTHER THAN hERs?

In this thesis the activity towards hERα and hERβ was determined for various (prenylated) flavonoids and isoflavonoids (**Chapters 3** and **4**). Nevertheless, other nuclear receptors are also present in the human body. It is known that (iso)flavonoids can bind (at least) to two of those receptors: the G-protein coupled estrogen receptor (GPER) and the androgen receptor (AR).[49] Hence, it is important to predict the influence of prenylation on the activity towards those receptors as well. In this way increased insight in *in vivo* activities might be obtained.

G-protein coupled estrogen receptor

The GPER has received relatively little attention so far, as it was evidenced only in 2000 that it was activated by E_2.[50] The activity of the GPER is most probably a rapid response that occurs within minutes after exposure to estrogenic compounds, in contrast to the slower transcriptional response upon activation of the hERs.[51]

Compounds with an agonistic mode of action towards hERs can also have an agonistic mode of action towards GPER, like 17β-estradiol and genistein (Figure 6.6).[52] There are also compounds that show opposite modes of action towards GPER and hERs.[52] For example, ICI 182,780, known as a full hERα antagonist, behaves as an GPER full agonist (Figure 6.6).[53] The synthetic compounds G1, G15 and G36 show almost no response towards hERs, whereas they have an agonistic (G1) or antagonistic (G15 and G36) mode of action towards GPER (Figure 6.6).[54,55] All compounds with an agonistic mode of action towards GPER contain an oxygen atom in their structure around the same position as the 17β-position of E_2 (Figure 6.6), whereas this oxygen is absent in all full GPER antagonists.[56] All flavonoids and isoflavonoids described in this thesis also have an oxygen atom around the same position as that of the 17β-position of E_2. So, it is speculated that these flavonoids and

isoflavonoids will act as GPER agonists. Prenylation will probably not modulate the activity towards the GPER to the same extent as towards hERs, as it was shown with *in silico* modelling that the apolar sidechain of tamoxifen (Figure 6.3) and ICI 182,780 (Figure 6.6) was accommodated in the large ligand binding cavity of the GPER.[56] Therefore, it is speculated that the prenyl groups will also fit in the GPER and that prenylated (iso)flavonoids will show an agonistic mode of action, as suggested for unprenylated (iso)flavonoids.[52] Nevertheless, the exact mode of action of (prenylated) (iso)flavonoids towards the GPER should be confirmed experimentally.

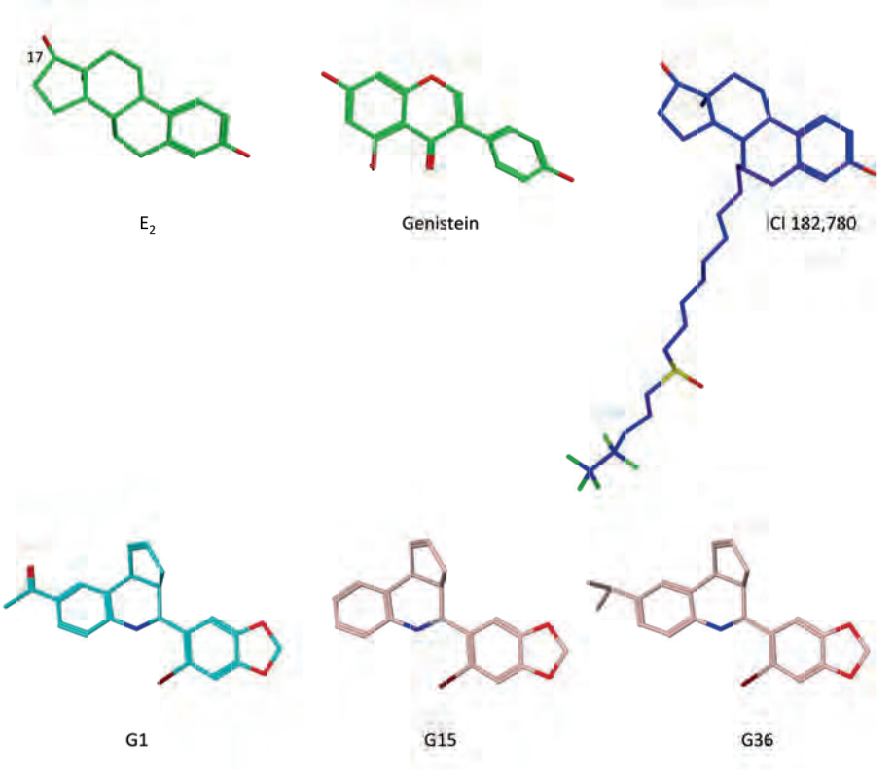

Figure 6.6. Minimalized molecular structures of agonists towards hER and GPER (indicated in green). Antagonists towards hER and agonist towards GPER (indicated in blue). No activity towards hER and agonist towards GPER (indicated in cyan). No activity towards hER and antagonistic towards GPER (indicated in pink).

Androgen receptor

It is known that some (iso)flavonoids with estrogenic activity can also bind to the androgen receptor (hAR)[49] and induce an antagonistic mode of action.[1,15,57] Genistein can inhibit cell proliferation of human prostate cells.[58] However, it is not certain whether this is due to its antagonistic mode of action towards the hAR, as the cell line which was used to study the effect also expressed other nuclear receptors. The influence of prenylation of compounds on their activity towards the androgen receptor is not yet known.

As an additional experiment, twelve flavonoids and isoflavonoids, with different backbone structures and prenylation, were tested[59] for their activities towards hAR (Table 6.4). Androgenicity of the compounds was tested as described in **Chapter 3** for hERα. Only four compounds could bind to hAR, and they all showed an antagonistic mode of action. The unprenylated isoflavene dehydroequol and the unprenylated isoflavan equol gave signal reductions of testosterone (T) of 100% and 89%, respectively (Table 6.4). It is known that these compounds can inhibit the growth of prostate cancer cells.[60,61] The single prenylated glabridin decreased the signal with 57% (Table 6.4). Addition of an extra methyl (4'-*O*-methyl-glabridin), methyl + hydroxyl (3'-hydroxy-4'-*O*-methyl-glabridin) or second prenyl group (hispaglabridin A and B) to glabridin resulted in loss of activity towards the hAR. It might be speculated that these compounds are too large to fit in the ligand binding domain of the hAR. Unprenylated and single prenylated flavanones did not show activity towards hAR, whereas the double prenylated flavanone glabrol showed a 78% signal reduction of T (Table 6.4). This is remarkable, as it was not expected that this relatively large flavonoid would fit in the ligand binding domain. Possibly, glabrol shows an antagonistic mode of action towards the hAR due to another mechanism than *e.g*. equol, like binding to the AF-2 co-activator site of hAR.[62] Based on the results obtained it is postulated that the mode of action towards the hAR was not modulated by prenylation, in contrast to hERs.

Table 6.4. Molecular characteristics and activity of compounds towards hAR as obtained in yeast androgen bioassay.

Trivial name	Molecular characteristics				Agonist activity	Antagonist activity		Overall activity
	1st prenyl	Pos.	2nd prenyl	Pos.	EC_{50} (nM)	Decrease (%)	Conc.[a] (nM)	
Isoflavenes								
Dehydroequol	-	-	-	-	n.r.	100	$1.0x10^5$	Antagonist
Glabrene	Pyran	δ	-	-	n.r.	n.r.	-	No response
Isoflavans								
Equol	-	-	-	-	n.r.	89	$1.0x10^5$	Antagonist
Glabridin	Pyran	α	-	-	n.r.	57	$1.0x10^4$	Antagonist
3'-hydroxy-4'-*O*-methyl-glabridin	Pyran	α	-	-	n.r.	n.r.	-	No response
4'-*O*-methyl-glabridin	Pyran	α	-	-	n.r.	n.r.	-	No response
Hispaglabridin A	Pyran	α	Chain	δ	n.r.	n.r.	-	No response
Hispaglabridin B	Pyran	α	Pyran	δ	n.r.	n.r.	-	No response
Flavanones								
Naringenin	-	-	-	-	n.r.	n.r.	-	No response
6-Prenylnaringenin	Chain	β	-	-	n.r.	n.r.	-	No response
8-Prenylnaringenin	Chain	α	-	-	n.r.	n.r.	-	No response
Glabrol	Chain	α	Chain	γ	n.r.	78	$1.0x10^5$	Antagonist

Androgenicity of the compounds is tested as described in **Chapter 3** for hERα.

n.r. = No response.

[a] Concentration at which the maximum decrease was reached.

METABOLISM OF PRENYLATED (ISO)FLAVONOIDS

Influence of metabolism on estrogenic activity

(Prenylated) flavonoids and isoflavonoids are extensively metabolized by phase I and II enzymes. Many hydroxylated isomers were observed after *in vitro* metabolism using pork S9 preparations (**Chapter 5**). The number of which increased with prenylation, especially with chain prenylated (iso)flavonoids. Moreover, the yield of glucuronidation was higher for prenylated than for unprenylated (iso)flavonoids (**Chapter 5**). For other estrogenic compounds, *e.g.* tamoxifen, it is known that metabolites resulting from phase I enzymes have modified estrogenicity compared to the parent compound.[63] For example, compared to tamoxifen, the phase I metabolites 4-hydroxy-tamoxifen (hydroxylation) and endoxifen (demethylation and hydroxylation) both have a 100-fold higher affinity for hERα, and are 30 and 100 times more potent, respectively, in suppressing cell proliferation.[64,65] This could be caused by the extra hydroxyl group, which might be able to make extra hydrogen bonds with the hER. In these examples, the mode of estrogenic action of the hydroxylated metabolites did not change, only the affinity (EC_{50}) increased. When comparing glyceofuran (a glyceollin with a hydroxyl group on the furan prenyl) with other furan prenylated glyceollins without a hydroxyl group on the furan prenyl (like glyceollins III and V), it appeared that the mode of action was changed by hydroxylation of the prenyl group (**Chapter 4**). It should be noted that the furan prenyl groups of glyceofuran, glyceollin III and V are not identical. They differ in the number and position of the double bonds in the furan prenyl moeiety. Nevertheless, it shows that hydroxylation on the prenyl group can have a large influence on estrogenicity.

Glucuronidation will probably increase the clearance from the human body more than hydroxylation, as hydroxylation alone might not increase polarity sufficiently enough for efficient clearance.[66] Moreover, glucuronidation will lead to inactive metabolites, as it was shown that glucuronidated metabolites of the isoflavones daidzein and genistein only become estrogenic after deconjugation.[67] In conclusion, more hydroxylated isomers and a lower glucuronidation yield were observed for prenylated (iso)flavonoids than for unprenylated (iso)flavonoids (**Chapter 5**). This might modulate their estrogenic activity and clearance from the human body.

For future research it is important to study bioavailability of prenylated flavonoids and isoflavonoids, together with potential colonic biotransformations. Prenylation increases the hydrophobicity of the compounds, leading to an increased affinity for biological membranes.[28] This suggests that prenylated flavonoids and

isoflavonoids have an increased bioavailability when passive absorption occurs. Moreover, it was observed that daidzein could be converted by intestinal microflora to the more active estrogen equol.[68] It cannot be excluded that intestinal microbiota perform additional modifications on prenyl substituents. So far, bioavailability and biotransformations of prenylated flavonoids and isoflavonoids has received little attention.

Preparation of *in vitro* phase I liver metabolites

From the above it is clear that it is important to know the estrogenicity of the hydroxylated metabolites. Hydroxylated metabolites can be synthesized, but this is a laborious task.[69] *In vitro* preparation by a S9 liver enzyme mixture, and subsequent purification of the metabolites, might be an alternative. However, the cost efficiency of this *in vitro* bioconversion procedure, as used in **Chapter 5**, is relatively low. The yield of metabolites could not be improved by adding extra S9 liver mixture, longer incubation times, extra NADPH or the addition of a fresh dose of S9 liver mixture (**Chapter 5**). Possibly, the molecules inhibit (some of) the cytochrome P450 enzymes, as has been shown for prenylated (iso)flavonoids.[70,71]

As an addition to those experiments mentioned in **Chapter 5**, it was afterwards tried to increase the cost efficiency by reducing the concentration of the expensive NADPH. Secondly, NADPH was replaced by different concentrations of the slightly less expensive $NADP^+$ in combination with glucose-6-phosphate (G6P). By using the action of the enzyme G6P-dehydrogenase on G6P, which is present in the liver S9 mixture, it is expected that $NADP^+$ will be converted to NADPH (Figure 6.7).

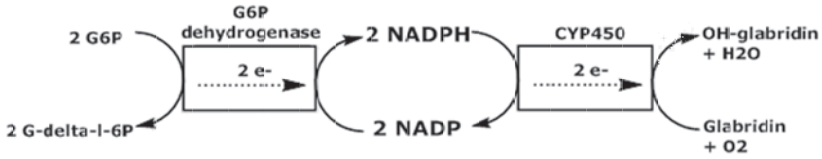

Figure 6.7. Reaction cascade of the regeneration and use of NADPH during metabolism.

In Figure 6.8, it can be observed that reduction of NADPH to 1 mM resulted in the same yield of hydroxy-glabridin isomer 1 as with 5 mM NADPH, whereas further reduction resulted in a decreased yield. The reduction from 5 mM to 1 mM NADPH will increase the cost efficiency with 76%. Additionally, the cost efficiency could slightly be increased when NADPH is replaced by NADP and G6P. It was observed that the replacement of NADPH with NADP and G6P gave the same yield of hydroxy-glabridin isomer 1 at concentrations similar or above 1 mM. Interestingly, at lower concentrations of NADP the yield decreased. This was unexpected as an excess of G6P

was added. The replacement of 5 mM NADPH by 1 mM NADP and 10 mM G6P will increase the total cost efficiency to 88%. It might enable future upscaling of the *in vitro* liver metabolism assay to obtain sufficient quantities of the metabolites for purification, in order to test their estrogenicty.

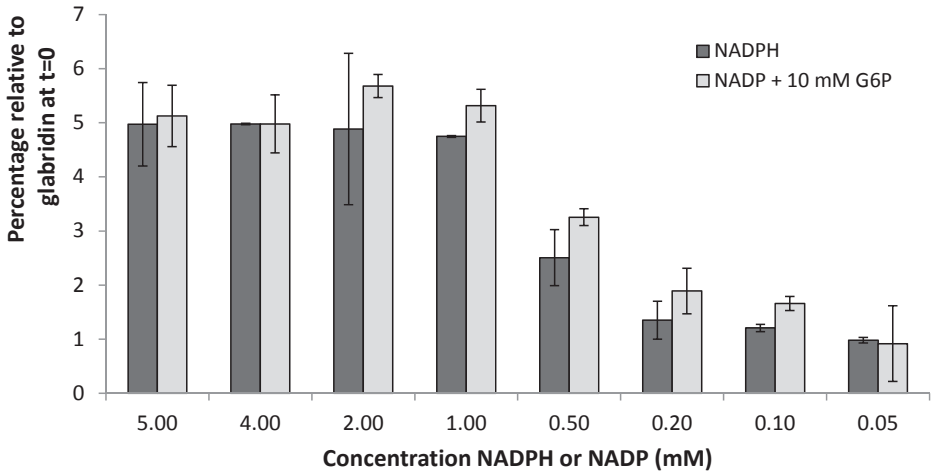

Figure 6.8. Proportion of hydroxy-glabridin isomer 1 formed upon decreasing NADPH concentrations and formed by replacement of NADPH with various concentrations of NADP and 10 mM G6P. The experimental set-up was similar to the one described in **Chapter 5**.

PURIFICATION AND CHARACTERIZATION OF PRENYLATED (ISO)FLAVONOIDS

Until now this **General Discussion** focused mainly on the estrogenic characteristics of prenylated (iso)flavonoids, which relates to the first aim of this thesis: the establishment of structure-function relationships. The purification and subsequent identification of 18 prenylated (iso)flavonoids was key to successful completion of that endeavor. Essential in this was the use of LC-MS for both purification and identification of the compounds. In establishing structure-metabolism relationships, the second aim of this thesis, LC-MS was once more essential.

Purification of prenylated (iso)flavonoids

Preparative HPLC is the method of choice for obtaining purified compounds. However, when a large array of closely resembling molecules, as is the case in our research, is present, usually 2D-HPLC-MS is performed. In our research we did not apply 2D-HPLC, but instead we used Flash chromatography as the first dimension in separation,

followed by preparative HPLC (**Chapters 3** and **4**). We are of the opinion that Flash chromatography is a very useful pre-purification step, as it enables a first separation between the different unprenylated and prenylated (iso)flavonoids. This was the case for all different (iso)flavonoids from the soybean (**Chapters 2** and **4**) as presented in Figure 2.5 of **Chapter 2**, in which the compositions of the different fractions are shown. The same procedure was used for the licorice extract (**Chapter 3**) of which the Flash chromatogram is shown in Figure 6.9. Clearly, with 2 different sets of molecules a good pre-fractionation could be obtained. In both cases, fractions with similar compositions were pooled to be further purified by preparative HPLC. This resulted in a more efficient and cost-effective purification procedure than when using preparative HPLC alone, as a Flash chromatography unit is less expensive than a preparative HPLC unit. Also, by applying pre-fractionation using Flash chromatography impurities fouling the preparative HPLC columns are removed, thereby extending the lifetime of expensive HPLC columns.

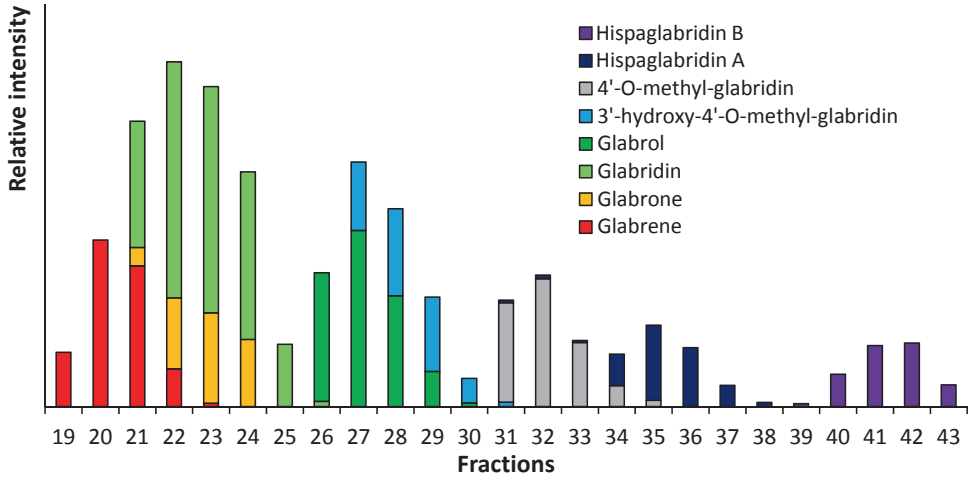

Figure 6.9. (Iso)flavonoid composition (based on UV_{280nm}) of Flash chromatography fractions of the licorice root extract by RP-UHPLC-MS.

Upon purification acidification of eluents is often used to improve chromatographic resolution. Nevertheless, this complicates the sample work-up downstream in the purification procedure. It was shown in **Chapter 2** that heat and acid could convert 6a-hydroxy-pterocarpans (glyceollins) into their respective 6a,11a-pterocarpenes (dehydroglyceollins), which influenced their estrogenic activity (**Chapters 2** and **4**). In this thesis the pools obtained after Flash chromatography or preparative HPLC contained prenylated flavonoids and isoflavonoids, water, ACN and traces of acetic

acid (HOAc) or formic acid (FA). The process used to obtain purified prenylated flavonoids and isoflavonoids includes evaporation of almost all solvents under vacuum (in case of Flash chromatography) or with a flow of N_2 gas (in case of preparative HPLC) at 40 °C. Subsequently, compounds were solubilized in *tert*-butanol, frozen and lyophilized. Sticky material was obtained if the water was not completely evaporated. This worked well for all prenyated flavonoid and isoflavonoid subclasses, except for 6a-hydroxy-pterocarpans, which were converted into 6a,11a-pterocarpenes. This conversion is most likely caused by gradually increasing acid concentrations upon evaporation of solvent, as organic solvents as ACN will evaporate prior to the acids. To overcome this problem, only organic solvents were evaporated and heat was omitted during evaporation. Afterwards, the pools containing the compounds, water and acid were frozen immediately with liquid nitrogen and lyophilized. After lyophilizing the pH was above 6. This material was solubilized in *tert*-butanol, frozen and lyophilized in order to obtain fluffy material. In conclusion, freeze drying after evaporation of the organic solvent was the most efficient way to remove HOAc and FA.

Characterization of prenylated (iso)flavonoids

Throughout this thesis research identification of prenylated (iso)flavonoids was mostly done with RP-UHPLC-ESI-MS. Nevertheless, NMR analysis of all purified compounds was performed to verify the annotation based on RP-UHPLC-ESI-MS. In almost all cases the results were unambiguous and the annotations based on UHPLC-MS were correct. In some cases NMR spectroscopy was needed: it was essential to make a distinction between (dehydro)glyceollidin I and II, and between (dehydro)glyceollin IV and VI, as annotation based on UHPLC was difficult. This was due to the fact that RDA-fragments in MS^n were expected to be similar. Not only MS was applied in UHPLC, but it was also used during preparative HPLC-MS which was used for obtaining purified fractions. The simultaneous monitoring of MS data of the fractions made pooling of fractions less troublesome than solely based on UV.

The verification by NMR spectroscopy also had another benefit. Frequently in literature, purity of compounds isolation by LC-MS is based on UV. In our research, the purity of the compounds was based on ^{1}H-NMR and not on UV signal, as it was shown that impurities present were not always visible at 280 nm. For example, 4'-*O*-methyl-glabridin showed a purity of 98% at 280 nm, whereas some impurities were visible in the NI and PI mode MS spectra (**Chapter 3**). ^{1}H-NMR gave a purity of 81%, which is in line with the presence of impurities observed in MS data, thereby overruling the UV response. As purities were measured with ^{1}H-NMR spectroscopy, the purity could be determined unambiguously. As in most cases high purities (>90%) were obtained, the

use of NMR spectroscopy enabled the establishment of several molecular extinction coefficients not presented before in literature (**Chapters 3** and **4**).

In **Chapter 5** *in vitro* liver metabolites were analyzed with RP-UHPLC-ESI-MS. Annotation of the metabolites could be achieved with MS^n spectra and a MS interpretation guideline to annotate the metabolites was made. Either purification of individual compounds, followed by NMR spectroscopy or LC-MS-NMR would enable verification of the annotations. However, this was beyond the scope of our research, considering the 100 metabolites observed. UV quantification (280 nm) of the sum of absorbances of the metabolites annotated led to an average difference of 15% in recovery compared to the decrease of absorbance of the parental compounds. Differences were expected as the molecular extinction coefficients of the metabolites formed are unknown. Besides, concentrations of metabolites below the detection limit might also underestimate the recovery. As it is not expected that the compounds were unstable at the experimental settings used, average yield of the different metabolites was based on the decrease of absorbance of the parent compound, rather than that of the sum of metabolites.

In conclusion, although laborious when sufficient material is needed for bioassays, NMR and the determination of molar extinction coefficients, the use of LC-MS for the purification and annotation of prenylated (iso)flavonoids can be regarded as rather straightforward without any large difficulties.

PROSPECTS

In this thesis it is shown that prenylation can modulate estrogenicity, *e.g.* chain prenylation of naringenin (to 8-prenylnaringenin) leads to an increased affinity towards the hERs, and it was observed that the pyran prenylated glabridin has an antagonistic mode of action, whereas unprenylated equol is agonistic. It was also shown that several purified glyceollins and dehydroglyceollins can act as SERMs or showed ER subtype-selective behavior, although at high concentrations (**Chapter 4**).

Consumption of (prenylated) flavonoids and isoflavonoids might result in beneficial health effects. Soybean extracts, which mainly contain compounds with an agonistic mode of action, are currently used as food supplements to prevent menopausal complaints.[60, 61] It is known that dietary agonistic estrogens, like soy isoflavones, are helpful to counteract the drop in endogenous estrogen levels in postmenopausal women.[72,73] Secondly, agonistic compounds can help to lower the incidence of osteoporosis and the risk of hip fracture by maintaining or modestly improving bone mass.[72,73] Germination and fungal elicitation of soybeans can increase the content of prenylated isoflavonoids, which yields extracts with more antagonistic compounds than those from untreated soybeans (**Chapters 3** and **4**). So, extracts with

unprenylated and prenylated (iso)flavonoids seem less promising to counteract menopausal complaints than extracts which contain unprenylated (iso)flavonoids.
For pharmaceutical applications, prenylated (iso)flavonoids might be of interest, as those compounds had specific and interesting SERM and ER subtype-selective behavior. Compounds with those characteristics might be used as natural lead molecules for the development of drugs for the treatment of, for example, breast cancer.

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Summary

The increased intake of unprenylated and prenylated (substitution with a C_5-isoprenoid group) flavonoids and isoflavonoids from natural sources is often correlated with a decreased risk on diseases related to a Western lifestyle. The possible interactions of these compounds with the human estrogen receptors, hERα and hERβ, are thought to be partly responsible for this beneficial effect. Prior to our research, it was shown that prenylation of (iso)flavonoids might modulate their estrogenic activity, but structure-activity relationships were not understood. The first aim of this thesis is to understand the structure-activity relationships of prenylated (iso)flavonoids towards the hERs. This was done by *in vitro* activity measurements in combination with *in silico* modelling. Moreover, for *in vivo* bioactivity, it is important to understand the metabolism of (prenylated) (iso)flavonoids, as metabolism can change their molecular structure and thus also influence their estrogenic properties. Therefore, the second aim of this thesis is to understand structure-metabolism relationships of unprenylated and prenylated (iso)flavonoids.

Chapter 1 provides an overview of the structural characteristics of (iso)flavonoids and indicates the main prenylated (iso)flavonoids present in licorice and challenged soybean seedlings. Also, the common methods to identify prenylated (iso)flavonoids are indicated. The mode of action via estrogen receptors and methods for measuring hER activation are explained. Furthermore, an overview of the mode of estrogenic action of 81 known (prenylated) phytoestrogens is presented. Finally, an overview of different metabolism pathways is given.

In **Chapter 2** it is shown that the isoflavonoid composition of an ethanolic extract of fungus-treated soybean sprouts was strongly altered by a combined acid/heat treatment. UHPLC-MS analysis showed that 6a-hydroxy-pterocarpans were completely converted into their respective, more stable, 6a,11a-pterocarpenes. Other (iso)flavonoids, from the isoflavone and coumestan subclasses, only showed a conversion of ~15%. Subsequently, mixtures enriched in prenylated 6a-hydroxy-pterocarpans (pools of glyceollin I/II/III and glyceollin IV/VI) or prenylated 6a,11a-pterocarpenes (pools of dehydroglyceollin I/II/III and dehydroglyceollin IV/VI) were purified, and tested for activity on both human estrogen receptors (hERα and hERβ). In particular, the response towards hERα changed, from an agonistic mode of action for glyceollins to an antagonistic mode of action for dehydroglyceollins. Towards hERβ, a decrease in agonistic activity was observed, but no change in mode of estrogenic action. These results indicate that the introduction of a double bond with the concomitant loss of a hydroxyl group in 6a-hydroxy-pterocarpans extensively modulates the estrogenic activity.

In **Chapter 3**, a set of 12 compounds was made, *i.e.* containing six prenylated (iso)flavonoids that were purified from a licorice root extract and six commercially available (iso)flavonoids. The agonistic and antagonistic activities of these compounds

towards both hERα and hERβ were determined. Differences in the modes of estrogenic action were observed for the various compounds tested. In general, each compound had the same mode of action towards both hERs. *In silico* modelling was performed in order to study the differences in estrogenicity observed between the compounds. This suggested that prenyl chains fit into the hydrophobic pocket present in the hER, which results in an increased agonistic activity. Besides, it was shown that an increase in length (~1.7 Å), as in pyran prenylated isoflavonoids, resulted in an antagonistic mode of action. This might be caused by collision of the pyran ring with helix 11 in the ligand binding cavity of the hER.

Seven prenylated 6a-hydroxy-pterocarpans and five prenylated 6a,11a-pterocarpenes with different kinds of prenylation were purified from an ethanolic extract of fungus-treated soybean sprouts using a three-step purification procedure (**Chapter 4**). The activity of these compounds towards hERα and hERβ was determined in yeast bioassays and the activity towards hERα was additionally tested in an U2-OS based hERα CALUX bioassay. In the yeast bioassay, compounds with chain prenylation showed in general an agonistic mode of action towards hERα, whereas furan and pyran prenylation mostly led to an antagonistic mode of action. Five of these antagonistic compounds had an agonistic mode of action in the U2-OS based hERα CALUX bioassay, implying that these compounds can act as SERMs. The yeast bioassay also identified eight ER subtype-selective compounds, with either an antagonistic mode of action or no response towards hERα and an agonistic mode of action towards hERβ. The ER subtype-selective compounds were characterized by a 6a-hydroxy-pterocarpan or 6a,11a-pterocarpene backbone structure. It is suggested that the extra D-ring or the length (12-13.5 Å) of these compounds cause the agonistic mode of action towards hERβ, thereby inducing ER subtype-selective behavior.

In **Chapter 5**, *in vitro* liver metabolism of eleven prenylated (iso)flavonoids was investigated by determining their phase I, glucuronyl and sulfate metabolites using pork liver preparations. Hundred metabolites were annotated using RP-UHPLC-ESI-MS^n. The positions of the hydroxyl groups attached during phase I metabolism were elucidated, considering their relevance for estrogenic activity. A mass spectrometry-based data interpretation guideline was proposed for identification of hydroxyl isomers. It was also found that phase II glucuronidation is the dominant route of modification for all compounds. In order to relate structure to metabolism, compounds were classified based on three criteria: backbone structure (isoflavene, isoflavan or flavanone), number of prenyl groups (0, 1 or 2), and prenyl configuration (chain or pyran). Glucuronidation was most extensive for isoflavenes and for unprenylated compounds (yield of 90-100%). Pyran and chain prenylation gave more complex hydroxylation patterns with 4 or more than 6 hydroxyl isomers, respectively,

compared to unprenylated compounds (only 1 hydroxyl isomer). Moreover, the number of hydroxyl isomers also increased with the number of prenyl groups.

Chapter 6 discusses the findings presented in this thesis, addressing structural characteristics influencing estrogenicity and comparing molecules with promising estrogenic behavior, found by us, with known estrogens from the literature. In addition, the activity of prenylated (iso)flavonoids towards the androgen and GPER receptors is evaluated. Also, methods used for fractionation and identification, and potential influence of metabolism on estrogenic activity are discussed. Finally, prospects for the use of prenylated (iso)flavonoids are given.

In conclusion, the mode of estrogenic action is modulated by kind of prenylation and the different modes of action are strongly correlated with conformational changes in the ligand-binding domain of hER. Furthermore, compounds with interesting SERM or ER subtype-selective behavior were identified. It was demonstrated that the kind of prenylation was most important for determining SERM activity, whereas additionally the backbone structure was of importance for determining ER subtype-selectivity. Interestingly, it was observed that prenylated (iso)flavonoids are differently metabolized as unprenylated (iso)flavonoids by phase I and II enzymes. All these findings can be useful for drug developers.

Samenvatting

De verhoogde inname van niet-geprenyleerde en geprenyleerde (substitutie met 5 koolstofatomen) flavonoïden en isoflavonoïden afkomstig uit natuurlijke bronnen wordt vaak gerelateerd aan een verlaagd risico op ziektes die geassocieerd worden met een westerse levensstijl. Er wordt gedacht dat de mogelijke interactie van deze componenten met de menselijke oestrogeenreceptoren, hERα and hERβ, zorgt voor deze positieve effecten. Voordat dit onderzoek startte, is al aangetoond dat geprenyleerde (iso)flavonoïden de oestrogene activiteit mogelijk kunnen veranderen, maar structuur-activiteits relaties werden niet begrepen. Het eerste doel van dit proefschrift is het begrijpen van structuur-activiteits relaties van geprenyleerde (iso)flavonoïden met betrekking tot de hERs. Dit werd gedaan met activiteitsmetingen *in vitro* (buiten het lichaam) in combinatie met modelleren *in silico* (computersimulatie). Voor bioactiviteit *in vivo* (in het lichaam), is het belangrijk om het metabolisme van (geprenyleerde) (iso)flavonoïden te begrijpen, omdat metabolisme (stofwisseling) de moleculaire structuur en dus ook de oestrogene activiteit kan beïnvloeden. Daarom is het tweede doel van dit proefschrift om structuur-metabolisme relaties van niet-geprenyleerde en geprenyleerde (iso)flavonoïden te begrijpen.

Hoofdstuk 1 geeft een overzicht van de structurele eigenschappen van (iso)flavonoïden en laat de belangrijkste geprenyleerde (iso)flavonoïden zien die aanwezig zijn in zoethout en gestreste sojakiemen. De veelgebruikte methodes om geprenyleerde (iso)flavonoïden te analyseren worden ook vermeld. De werking van de oestrogeenreceptoren en de methodes om oestrogene activiteit te meten worden uitgelegd. Daarnaast wordt er een overzicht gepresenteerd met de oestrogene activiteit van 81 bekende (geprenyleerde) oestrogenen. Tot slot wordt er een overzicht gegeven van de mogelijke metabolische reacties.

In **Hoofdstuk 2** wordt beschreven dat de samenstelling van isoflavonoïden in een ethanolextract van met schimmel gestreste sojakiemen ernstig verandert door een combinatie van zuur en hitte. UHPLC-MS analyse toonde aan dat 6a-hydroxy-pterocarpanen compleet omgezet worden in de meer stabiele 6a,11a-pterocarpenen. Andere (iso)flavonoïden, die behoren tot de isoflavon en coumestaan subklassen, worden voor maar ongeveer vijftien procent omgezet. Vervolgens zijn er mengels gezuiverd die verrijkt waren in geprenyleerde 6a-hydroxy-pterocarpanen (fracties met glyceollin I/II/III en glyceollin IV/VI) of geprenyleerde 6a,11a-pterocarpenen (fracties met dehydroglyceollin I/II/III en dehydroglyceollin IV/VI). Deze mengels zijn getest op hun oestrogene activiteit op beide menselijke oestrogeenreceptoren (hERα en hERβ). Vooral de respons op hERα wordt veranderd, glyceollins gedragen zich als agonist (hezelfde als oestrogeen) terwijl dehdyroglyceollins zich gedragen als antagonist (tegenovergestelde van oestrogeen). Als er naar hERβ wordt gekeken, kan er een verlaging van de agonist activiteit worden gezien, maar een verandering van

agonist naar antagonist wordt niet gezien. Deze resultaten tonen aan dat de extra dubbele binding met tegelijkertijd het verlies van de hydroxyl groep in 6a-hydroxy-pterocarpanen, om zo 6a,11a-pterocarpenen te vormen, leidt tot een heel andere oestrogene activiteit.

In **Hoofdstuk 3** is een set van twaalf componenten gemaakt, bestaande uit zes geprenyleerde (iso)flavonoïden die zijn gezuiverd uit een zoethoutwortelextract en zes commercieel verkrijgbare (iso)flavonoïden. De agonist en antagonist activiteit van deze componenten op hERα en hERβ zijn bepaald. De verschillende componenten hadden een verschillende oestrogene werking (agonist danwel antagonist). Over het algemeen hadden alle componenten eenzelfde werking op beide hERs. *In silico* modelleren werd gebruikt om de verschillen in werking te bestuderen. Het is gesuggereerd dat een prenylketen in een hydrofobe pocket past die aanwezig is in de hER. Dit resulteert in een agonitische activiteit. Daarnaast werd aangetoond dat de verlenging (~1.7 Å) van de componenten, zoals bij isoflavonoïden geprenyleerd met een pyraan (een ring-gesloten keten; zesring), resulteerde in een antagonische activiteit. Dit kan veroorzaakt worden doordat de pyraanring botst met helix 11 in de ligand binding domein (waar een component bindt aan de receptor) van de hERs.

Acht geprenyleerde 6a-hydroxy-pterocapanen en vijf geprenyleerde 6a,11a-pterocarpenen met verschillende soorten prenylering zijn gezuiverd met behulp van een driestaps zuiveringsproces uit een ethanolextract van met schimmel gestreste sojakiemen (**Hoofdstuk 4**). De oestrogene activiteit van deze componenten op hERα en hERβ is bepaald met een gistmethode en de activiteit op de hERα is ook nog getest met behulp van een op U2-OS (botcellen) gebaseerde hERα CALUX-methode. Componenten met ketenprenylering lieten in het algemeen een agonistische activiteit zien op de hERα in de gistmethode, terwijl furaan- (een ring gesloten keten; vijfring) of pyraan-prenylering leidde tot een antagonistische activiteit. Vijf van deze antagonistische componenten hadden een agonistische activiteit in de op U2-OS gebaseerde hERα CALUX-methode. Dit suggereert dat deze componenten werken als SERMs (Selectieve Oestrogeen Receptor Modulator; stoffen met verschillende werking in verschillende weefsels). Met de gistmethode werden ook acht ER subtype-selectieve componenten (verschillend in werking op hERα en op hERβ), met een antagonistische activiteit of geen respons op hERα en een agonistische activiteit op hERβ. De ER subtype-selectieve componenten hebben allemaal een 6a-hydroxy-pterocarpaan of 6a,11a-pterocarpeen ruggengraatstructuur. Wij vermoeden dat de extra D-ring of de lengte (12-13.5 Å) van deze componenten de agonistische activiteit naar hERβ veroorzaakt en op die manier dus ook ER subtype-selectiviteit.

In **Hoofdstuk 5** wordt *in vitro* levermetabolisme van elf geprenyleerde (iso)flavonoïden onderzocht. Dit werd gedaan door te kijken naar de fase I (+hydroxyl), glucuronyl (+glucuronzuur) en sulfaatmetabolieten (stofwisselings

producten), die gemaakt werden met behulp van varkensleverpreparaties. Honderd metabolieten konden worden geannoteerd met behulp van RP-UHPLC-ESI-MS^n. De positie van de hydroxylgroepen die tijdens fase I metabolisme aan het molecuul vast komen te zitten, kon worden bepaald. Een op massa spectrometrie gebaseerd interpretatieschema wordt voorgesteld om de hydroxylmetabolieten te identificeren. Wij ontdekten dat glucuronidering de dominante route van metabolisme was voor alle componenten. Om structuur-metabolisme relaties te maken, werden de componenten ingedeeld op basis van drie criteria: ruggengraatstructuur (isoflaveen, isoflavan of flavanon), aantal prenylgroepen (0, 1 of 2) en prenylconfiguratie (keten of pyraan). Glucuronidering was het meest aanwezig bij isoflavenen en bij niet-geprenyleerde componenten (opbrengst van 90-100%). Pyraan en ketenprenylering zorgden voor een meer complex hydroxylatiepatroon van vier tot meer dan zes hydroxylisomeren (dezelfde structuur formule, andere plaats van de hydroxylgroep), vergeleken met slechts één hydroxylisomeer voor de niet-geprenyleerde componenten. Bovendien, was het aantal hydroxylisomeren ook hoger als een component meer prenylgroepen bevatte.

In **Hoofdstuk 6** worden de belangrijkste bevindingen van deze thesis bediscussieerd. De structurele karakteristieken die de oestrogene werking beïnvloeden, worden besproken en daarnaast worden de componenten uit dit onderzoek vergeleken met bekende oestrogene componenten uit de literatuur. Eveneens wordt de mogelijke activiteit van geprenyleerde (iso)flavonoïden op de androgeen receptor en de GPER geëvalueerd. Methodes die gebruikt werden voor de zuivering en identificatie, en de mogelijke invloeden van metabolisme op de oestrogene activiteit, worden ook bediscussieerd. Uiteindelijk wordt gespeculeerd over de vooruitzichten voor het gebruik van geprenyleerde (iso)flavonoïden.

Concluderend, wordt de oestrogene activiteit beïnvloed door de soort prenylering en deze activiteit is sterk gecorreleerd aan veranderingen in de ligand-binding domein van de hER. Verder zijn er componenten met het interessante SERM of ER subtype-selectief gedrag geïdentificeerd. Uit dit onderzoek is gebleken dat het type prenylering het belangrijkste is voor de bepaling van SERM gedrag, terwijl de ruggengraatstructuur ook belangrijk is voor de bepaling van ER subtype-selectief gedrag. We hebben daarnaast ook geobserveerd dat geprenyleerde (iso)flavonoïden anders gemetaboliseerd worden door fase I en II enzymen dan niet-geprenyleerde (iso)flavonoïden. Al deze bevindingen kunnen bruikbaar zijn voor de farmaceutische industrie.

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Milou

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CURRICULUM VITAE

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LIST OF PUBLICATIONS

Van de Schans, M.G.M.; Vincken, J-P.; Bovee, T.F.H.; David Cervantes, A.; Logtenberg, M.J.; Gruppen, H. Structural changes of 6a-hydroxy-pterocarpans upon heating modulate their estrogenicity. *J. Agric. Food Chem.* **2014**, 62, 10475-10484.

Van de Schans, M.G.M.; Ritschel, T.; Bovee, T.F.H.; Sanders, M.G.; de Waard, P.; Gruppen, H.; Vincken, J-P. Involvement of a hydrophobic pocket and helix 11 in determining the mode of action of prenylated flavonoids and isoflavonoids in the human estrogen receptor. *Submitted for publication*.

Van de Schans, M.G.M.; Vincken, J-P.; de Waard, P.; Hamers, A.; Bovee, T.F.H.; Gruppen, H. Glyceollins and dehydroglyceollins isolated from soybean act as SERMs and ER subtype-selective phytoestrogens. *Submitted for publication*.

Van de Schans, M.G.M.; Bovee, T.F.H.; Stoopen, G.M.; Lorist, M.; Gruppen, H.; Vincken, J-P. Prenylation and backbone structure of flavonoids and isoflavonoids influence their phase I and II metabolism. *Submitted for publication*.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Courses

- Nuclear receptors in nutrition (NuGO eLearning), Wageningen, The Netherlands, 2011
- Food and biorefinery enzymology (VLAG), Wageningen, The Netherlands, 2011
- Chemical discovery and design course (CMBI), Nijmegen, The Netherlands, 2012
- Pharmacological aspects of nutrition (HNE), Wageningen, The Netherlands, 2014

Conferences

- GPCR day, Vlaardingen, The Netherlands, 2011
- 9th International conference on chemical structures, Noordwijkerhout, The Netherlands
- 26th International conference on polyphenols, Florence, Italy, 2012
- 7th World congress on polyphenol applications, Bonn, Germany, 2013[a]
- 27th International conference on polyphenols, Nagoya, Japan, 2014[b]

General courses

- VLAG PhD introduction week (VLAG), 2011
- PhD competence assessment (WGS), 2011
- Interpersonal communication for PhD students (WGS), 2012
- Project and time management (WGS), 2012
- Teaching and supervising thesis students, (WUR), 2012
- First Certificate in English (WUR In'to Languages), 2012-2013
- Techniques for writing and presenting a scientific paper (WGS), 2013

Additional activities

- Preparation PhD research proposal
- PhD trip FCH to Singapore and Malaysia, 2012[a,b]
- PhD trip FCH to Germany, Denmark and Finland, 2014[a,b]
- BSc/MSc thesis student presentations and colloquia, 2011-2015
- PhD presentations Food Chemistry, 2011-2015

[a] Poster; [b] Oral presentation
NuGO: Nutrigenomics Organization
VLAG: Graduate school for Nutrition, Food Technology, Agrobiotechnology and Health Sciences
CMBI: Centre for Molecular and Biomolecular Informatics
HNE: Human Nutrition
WGS: Wageningen Graduate School
WUR: Wageningen University and Research Centre
FCH: Food Chemistry

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Milou G.M. van de Schans, 2015