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Molecules of Interest

Xanthohumol and related prenylflavonoids from hops and beer: to your good health!

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Abstract

Xanthohumol (3′-[3,3-dimethyl allyl]-2′,4′,4-trihydroxy-6′-methoxychalcone) is the principal prenylated flavonoid of the female inflorescences of the hop plant ('hops'), an ingredient of beer. Human exposure to xanthohumol and related prenylflavonoids, such as 8-prenylnaringenin and isoxanthohumol, is primarily through beer consumption. Xanthohumol has been characterized a 'broad-spectrum' cancer chemopreventive agent in in vitro studies, while 8-prenylnaringenin enjoys fame as the most potent phytoestrogen known to date. These biological activities suggest that prenylflavonoids from hops have potential for application in cancer prevention programs and in prevention or treatment of (post-)menopausal 'hot flashes' and osteoporosis. Xanthohumol and 8-prenylnaringenin are metabolized into many flavonoid derivatives with modified 3,3-dimethyl allyl (prenyl) moieties. Xanthohumol is formed in lupulin glands by a specialized branch of flavonoid biosynthesis that involves prenylation and O-methylation of the polyketide intermediate chalconaringenin. Although a lupulin gland-specific chalcone synthase is known, the aromatic prenyltransferase and *O*-methyltransferase participating in xanthohumol have not been identified. The prenylflavonoid pathway is a possible target for breeding or biotechnological modification of hops with the aim of increasing xanthohumol levels for beer brewing and 8-prenylnaringenin levels for pharmaceutical production.

Keywords: Xanthohumol; 8-Prenylnaringenin; Prenylflavonoids; Flavonoids; Hops; *Humulus lupulus*; Beer; Antioxidant; Cancer chemoprevention; Phytoestrogen; Breast enhancement

1. Introduction

Xanthohumol is a structurally simple prenylated chalcone that occurs only in the hop plant, *Humulus lupulus* L. (Cannabaceae), where it is the principal prenylflavonoid of the female inflorescences (usually referred to as 'hops' or 'hop cones'). Hops are used to add bitterness and flavor to beer, and therefore the main dietary source of xanthohumol and related prenylflavonoids is beer. Xanthohumol and other prenylated chalcones have received much attention in recent years as cancer chemopreventive agents, while 8-prenylnaringenin, an isomerization product of desmethylxanthohumol also present in beer, enjoys fame as the most potent phytoestrogen isolated to date. These exciting biological activities may lead to biomedical application of xanthohumol and 8-prenylnaringenin in the future. Furthermore, hop prenylflavonoids are currently tested in many academic and industrial laboratories for other biological activities. At the same time, hop-containing herbal preparations are being marketed for breast enlargement in women, without proper testing of efficacy and toxicity. The use of hops in herbal preparations represents a small market and does not seem to be promoted by the hop growing industry. However, hop growers are very interested in alternative applications for hops and its constituents in view of the continuing trend of

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decreasing market prices of hops and improving agricultural production methods.

The purpose of this review is to provide an overview of the chemistry, biological activities, and biotechnological aspects of xanthohumol and other prenylated flavonoids from hops. Biosynthetically related to the prenylated flavonoids are the prenylated acylphloroglucinols, i.e., humulones (α-acids) and lupulones (β-acids), which represent the commercial value of hops as bittering substances for the beer industry but potential biomedical applications have not yet been identified for this group of terpenophenolics. Hops are also rich sources of flavonol glycosides and condensed tannins: these groups of flavonoids will not be treated either as they are widely distributed in the plant kingdom.

2. Distribution and chemotaxonomic significance of xanthohumol and related flavonoids

The distribution of xanthohumol is limited to and ubiquitous within *H. lupulus*. Xanthohumol is secreted as part of the hop resin ('lupulin') by glandular trichomes found on the adaxial surfaces of cone bracts. It is also found in the trichomes on the underside of young leaves. Xanthohumol is the main prenylflavonoid of hops (0.1–1% on dry weight); in the resin, xanthohumol is accompanied by at least 13 related chalcones (Table 1), all of which occur at 10–100-fold lower concentrations relative to xanthohumol. Most of the chalcones contain a free 2′-hydroxy group and can therefore isomerize to their corresponding flavanones. The structures of the most abundant prenylated hop flavanones, i.e., isoxanthohumol, 6-prenylnaringenin, and 8-prenylnaringenin, are also listed in Table 1.

In a study to determine whether prenylflavonoid variation had diagnostic value in distinguishing between hop cultivars, we found that the presence of xanthogalenol (Table 1), named after its source *H. lupulus* cv. 'Galena', was strictly limited to a few varieties that were all derived from *H. lupulus* cv. 'Brewer's Gold'. The latter variety is the result of an open-pollination event between a wild female plant from Manitoba and an unknown European male at Wye College, England, in 1918 (Neve, 1991). This interesting piece of information led us to include many wild North American hop plants and one sample of *Humulus cordifolius* (originally from Japan) in our survey of 22 flavonoids. Multivariate analysis of the flavonoid profiles obtained from 120 cone and leaf samples yielded two well-resolved groups of hops: (1) European hops and (2) Central US and Japanese wild hops (Stevens et al., 2000). This dichotomy with regard to the presence of xanthogalenol and other 4′-O-methylated chalcones clearly supports the hypothesis that the present-day disjunct distribution of *H. lupulus* is the result of eastward migration to Japan and America and westward to Europe from the center of the species' origin in China during the mid to late Tertiary (Small, 1980; Neve, 1991).

3. Isolation and chemical synthesis

Xanthohumol was first isolated, partially characterized and named by Power et al. (1913). They also obtained a related substance which they called humulol, which was later identified as the flavanone, isoxanthohumol, by Verzele et al. (1957). In the years following 1957, there remained some doubt as to the position of the prenyl substituent relative to the methoxy group in xanthohumol. Two independent groups were finally able to confirm Verzele's original structure as the correct structure of xanthohumol by partial synthesis and chemical degradation studies (Vandewalle, 1961; Orth and Riedl, 1963). At the time of preparation of this review, no formal synthesis of xanthohumol could be retrieved from the SciFinder database. The lack of interest in the synthesis of this compound is probably related to the availability of xanthohumol from sources such as CO_2-extracted hops, a waste product of the hop-processing industry. It is expected that new synthetic methods will appear in the literature when labeled xanthohumol is needed for in vivo studies. The recent interest in 8-prenylnaringenin as a novel phytoestrogen has spurred the development of a synthesis for 8-prenylnaringenin from naringenin and prenyl alcohol (Gester et al., 2001).

The external accumulation of xanthohumol and related prenylflavonoids in lupulin glands significantly facilitates the extraction of these lipophilic compounds (Stevens et al., 1997). Whole hop cones can be rinsed briefly with chloroform or acetone to dissolve the resin constituents. The extract thus obtained contains the bulk of the bitter acids and the prenylflavonoids but is largely free of cone tissue constituents. Crude xanthohumol can be isolated from the resin extract by column chromatography on Sephadex LH-20 using methanol as the eluent. The crude product can be purified by semi-preparative HPLC on a reversed-phase C_{18}-column. This method has been improved in recent years by extracting hops with acetone after pre-extraction of hops using supercritical CO_2 to remove the bulk of the bitter acids (supercritical CO_2-extraction is an industrial process for the production of bitter acids; the wasted hop materials were made available to us). Addition of a 5% solution of sodium carbonate in water to the concentrated acetone extract usually results in precipitation of chlorophyll and other fatty materials. The aqueous layer, containing the prenylflavonoids in their phenolate form, is acidified and extracted with ethyl

Table 1
Prenylated chalcones and flavanones from hops

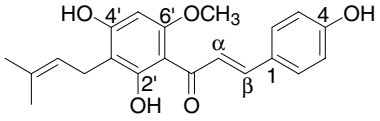
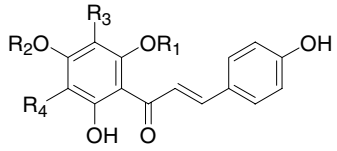
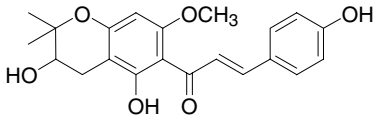
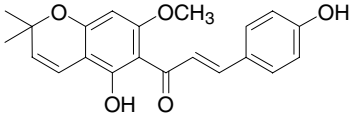
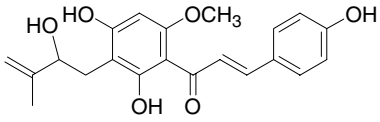
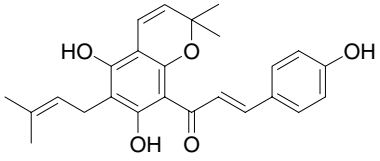
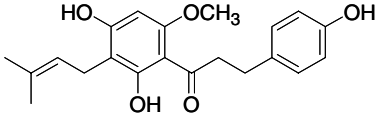
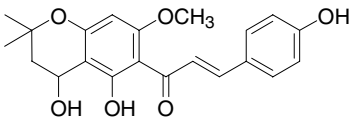
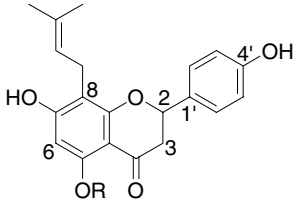
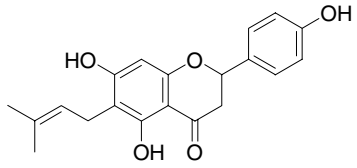
Structure	Trivial name	Reference
Chalcones		
	Xanthohumol	Power et al. (1913) Verzele et al. (1957) Huebner and Riedl (1960) Haensel and Schulz (1988)
		
R_1, R_2, R_3 = H, R_4 = Prenyl	Desmethylxanthohumol	Haensel and Schulz (1988)
R_1, R_3 = H; R_2 = Me, R_4 = Prenyl	Xanthogalenol	Stevens et al. (2000)
R_1, R_2 = Me; R_3 = H, R_4 = Prenyl	4′-*O*-Methylxanthohumol	Sun et al. (1989)
R_1, R_2, R_3 = H, R_4 = Geranyl	3′-Geranylchalconaringenin	Stevens et al. (1997)
R_1, R_2 = H; R_3, R_4 = Prenyl	3′,5′-Diprenylchalconaringenin	Stevens et al. (2000)
R_1 = Me, R_2 = H; R_3, R_4 = Prenyl	5′-Prenylxanthohumol	Stevens et al. (1997)
R_1, R_2 = Me; R_3, R_4 = H	Flavokawin	Stevens et al. (2000)
	Xanthohumol B	Tabata et al. (1997) Stevens et al. (1997)
	Xanthohumol C	Stevens et al. (2000)
	Xanthohumol D	Stevens et al. (2000)
	Xanthohumol E	Stevens et al. (2000)
	α,β-Dihydroxanthohumol	Etteldorf et al. (1999)
	*iso*-Dehydrocycloxanthohumol hydrate	Etteldorf et al. (1999)
Flavanones		
	R = H, 8-Prenylnaringenin R = Me, Isoxanthohumol	Mizobuchi and Sato (1984) Verzele et al. (1957)

Table 1 (*continued*)

Structure	Trivial name	Reference
	6-Prenylnaringenin	Mizobuchi and Sato (1984)
Other prenylated flavanones		Stevens et al. (2000)

acetate to recover the prenylflavonoids. One or more chromatographic steps are still necessary at this point to separate xanthohumol from other, minor prenylated flavonoids.

4. Dietary exposure

Beer is the most important dietary source of xanthohumol and related prenylflavonoids. The average person in the United States consumed 225 mL of beer per day in 2001 (USDA, 2003). Based on the assumption that this amount was consumed as US major brand lager/pilsner beers, the daily intake of total prenylflavonoids would be about 0.14 mg, with isoxanthohumol being the principal dietary prenylflavonoid (Table 2). Although xanthohumol is the major prenylflavonoid in hops, it is generally a minor prenylflavonoid in beer due to thermal isomerization of chalcones into flavanones during the brewing process. Xanthohumol has one free hydroxyl group that can participate in cyclization and therefore yields only one flavanone on cyclization, i.e., isoxanthohumol, while desmethylxanthohumol with two free *ortho* hydroxyl groups relative to C-1′ isomerizes into 6- and 8-prenylnaringenin. Relatively high levels of xanthohumol in beer usually point to a second, late addition of hops to the boiling wort (Stevens et al., 1999a). The daily intake of prenylflavonoids (ca. 0.14 mg) is relatively small compared to total polyphenols from beer (42 mg as catechin equivalents per day (Vinson et al., 2003)), from which one might conclude that prenylflavonoids contribute little to the antioxidant properties of beer. On the other hand, prenylflavonoids differ from other beer polyphenols, such as proanthocyanidins, flavonol glycosides, and phenolic acids, in that they are more lipophilic and therefore may be more effective antioxidants at lipophilic surfaces such as membranes and low-density lipoprotein (Stevens et al., 2003).

The presence of 8-prenylnaringenin in beer is due to isomerization of desmethylxanthohumol in the brew kettle. This prenylated flavanone is the most potent phytoestrogen to date (Milligan et al., 1999) and this has resulted in concerns as to the estrogenic effects of beer consumption. The daily intake of 8-prenylnaringenin through beer per capita in the US ranges from 3.3 (major brand lager/pilsner beers) to 54 μg (ales, porters and stouts). It is generally believed that this amount has no detrimental health effects due to the estrogenic effect of this phytoestrogen (see below).

5. Bioavailability and metabolism

Absorption, metabolism, and renal excretion of xanthohumol were studied in rats by Yilmazer (2001). After oral administration of xanthohumol to male rats (50 mg/kg), xanthohumol was detected in plasma mainly in the form of two mono-glucuronides whose maximum concentrations reached 180 and 65 nM after 4 h. The cumulative amounts of both xanthohumol glucuronides excreted in the urine reached a plateau at 12 h after oral administration and accounted for 0.3% and 0.05% of the administered dose. The recovery of unchanged xanthohumol from the urine was 0.2%. Other metabolites of xanthohumol, formed by cytochrome P450 enzymes, were detected in plasma and urine samples at much lower levels than the glucuronides (Yilmazer, 2001). These findings suggest that xanthohumol's bioavailability, defined as the fraction of the administered dose that is ultimately absorbed intact, is very low, possibly due to extensive intestinal metabolism by gut microorganisms.

No published data were available with regard to the bioavailability of isoxanthohumol and 8-prenylnaringenin at the time of preparation of this paper. In view of the increasing interest in 8-prenylnaringenin as a novel phytoestrogen, it is anticipated that pharmacokinetic data will appear in the literature within the next one or two years. Pharmacokinetic data are also urgently needed to assess the health risks of herbal supplements that contain high amounts of hop prenylflavonoids. This area of research has not progressed as much as studies of other phytoestrogens such as the soy isoflavones (Dixon and Ferreira, 2002).

A few studies have reported on the in vitro metabolism of xanthohumol and 8-prenylnaringenin using liver microsome preparations (Table 3). Buhler and coworkers studied the biotransformation of xanthohumol

Table 2
Prenylflavonoid contents in hops and beer measured by LC–MS/MS [adapted from (Stevens et al., 1999b)]

	Xanthohumol	Isoxanthohumol	8-Prenylnaringenin	Desmethylxanthohumol
Hop cones (% dry wt):	0.48[a]	0.008	0.002	0.12
Beer (μg/L)[b]:	Xanthohumol	Isoxanthohumol	8-Prenylnaringenin[d]	Total[c]
US major brand				
Lager/pilsner	34	500	13	590
Lager/pilsner	9	680	14	750
Lager/pilsner	14	400	17	460
Lager/pilsner	–	–	–	–
Northwest/US microbrews				
American porter	690	1330	240	2900
American hefeweizen	5	300	8	330
Strong ale	240	3440	110	4000
India pale ale	160	800	39	1160
Imported beers				
European stout	340	2100	69	2680
European lager	2	40	1	43
European pilsner	28	570	21	680
European pilsner	12	1060	8	1100
Other				
Non-alcohol beer	3	110	3	120

[a] The content of xanthohumol in hops can vary from 0.1% or less for aged hops to over 1% for high xanthohumol-producing varieties.
[b] Most beers contain no desmethylxanthohumol due to thermal isomerization in the brew kettle.
[c] Minor prenylflavonoids contributing to the total include 6-prenylnaringenin and 6-/8-geranylnaringenin.
[d] Tekel and co-workers developed a GC–MS method for analysis of 8-prenylnaringenin in beer and found concentrations ranging from 5 (limit of quantitation) to 19.8 μg/L in Belgian and other beers (Tekel et al., 1999).

by rat liver microsomes (Yilmazer et al., 2001b). The major metabolites appeared to be derived from the epoxide intermediate a, thus giving rise to the cyclic xanthohumol derivatives b and c by intramolecular nucleophilic attack (Table 3). Metabolite d is the dehydration product of metabolite c; these products were identical with xanthohumols B and C, respectively, isolated from hops (Stevens et al., 2000). Aromatic hydroxylation of the B ring of xanthohumol was also observed (Yilmazer et al., 2001b). In a subsequent study, Yilmazer et al. (2001a) studied the in vitro metabolism of xanthohumol by rat and human liver microsomes in the presence of uridine 5′-diphosphoglucuronic acid. Four glucuronic acid metabolites were detected by LC–MS, of which the two major metabolites were identified as the 4′-*O* and 4-*O*-glucuronides of xanthohumol.

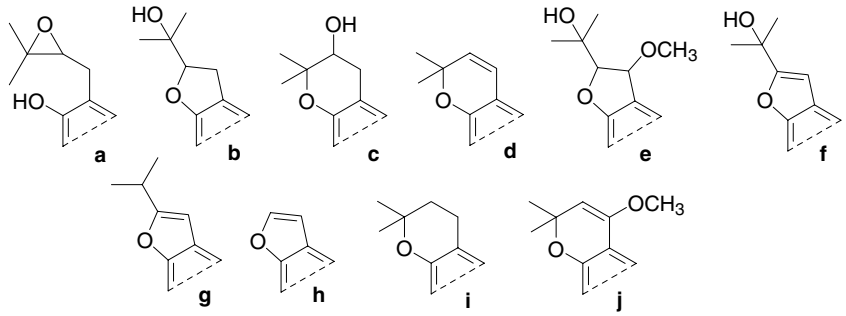
Van Breemen and co-workers (Nikolic et al., 2004) studied the metabolism of 8-prenylnaringenin by human liver microsomes and characterized 11 metabolites, four of which were identified as products derived from allylic radical intermediates (k–n), three (b–d) were formed from the epoxide intermediate a, and four other metabolites were identified as the flavone, dihydroflavonol, chromone, and B-ring hydroxylated analogs of 8-prenylnaringenin. In this study, oxidation of the prenyl methyl group was the predominant pathway (metabolites l–n), whereas none of these products were detected in Yilmazer's study (2001b), which was attributed by the van Breemen group to the much higher substrate concentration used in Yilmazer's work. Nikolic et al. (2004) found the same metabolites in incubations with rat liver microsomes, thus ruling out the possibility of species differences.

The microbial transformation of xanthohumol with the yeast, *Pichia membranifaciens* (ATCC 2254) yielded metabolites b, its flavanone isomer, and metabolite p (Herath et al., 2003b). In a subsequent study, these authors obtained metabolites l and m when xanthohumol was added to a culture of the fungus, *Cunninghamella echinulata* (NRRL 3655). The aim of this study was to generate mammalian metabolites for studies of cytotoxicity toward mammalian cell lines and for testing of their antimicrobial and antimalarial potential (Herath et al., 2003a).

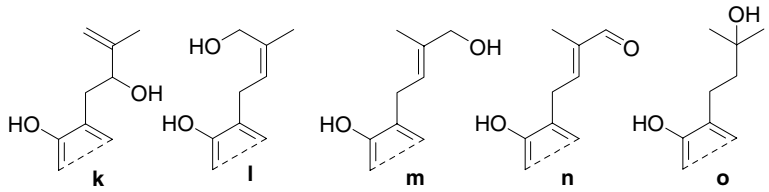
A detailed in vivo study of xanthohumol metabolism was published very recently by Nookandeh et al. (2004). These authors fed xanthohumol to 12 rats in a dose of 1000 mg/kg body weight, and 22 metabolites were isolated from the faeces and characterized by spectroscopic methods. Xanthohumol was largely recovered in unchanged form (89% of the total of recovered flavonoids), while the remainder consisted of metabolites that were isolated in microgram quantities only. Of the 22 metabolites, eight metabolites contained cyclic prenyl substituents involving the hydroxy group at C-4′ (b, d, e–j), five contained cyclic prenyl substituents with the

Table 3
Metabolites of xanthohumol and 8-prenylnaringenin identified in vitro and in vivo[a]

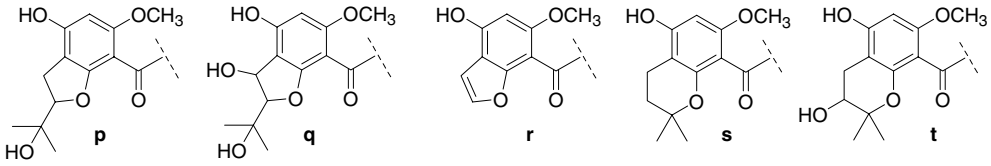
(A) Cyclic prenyl moieties with the oxygen at C-4′ (xanthohumol) or at C-7 (8-prenylnaringenin) being part of a ring system



(B) Metabolites with non-cyclic oxygenated prenyl substituents



(C) Metabolites with cyclic prenyl substituents that include the oxygen atom at C-2′ of xanthohumol



[a] For references to metabolites a–t, see text.

oxygen at C-2′ as the hetero atom (p–t), and three metabolites contained non-cyclic oxygenated prenyl substituents (k, l, and o). The remaining metabolites were identified as isoxanthohumol, the isoxanthohumol analog of metabolite o, xanthohumol-4′-*O*-glucuronide, xanthohumol-4-*O*-methyl ether and xanthohumol-4-*O*-acetate, and the α,β-epoxide of xanthohumol.

6. Cancer-related bioactivities

Cancer chemoprevention is different from cancer treatment in that it is aimed at modulation of pathways that are relevant to carcinogenesis with the effect that tumor formation is inhibited. Ideally, cancer prevention is achieved by long-term exposure to non-toxic agents, preferably as part of certain food products. Xanthohumol is the best studied cancer chemopreventive phytochemical isolated from hops. We show in the following paragraphs that xanthohumol is a 'broad-spectrum' cancer chemopreventive agent that acts by: (1) inhibiting the metabolic activation of procarcinogens, (2) induction carcinogen-detoxifying enzymes, and (3) by inhibition of tumor growth at an early stage.

Cancer-related studies of prenylflavonoids from hops have mainly focused on the in vitro activities of xanthohumol as a cancer chemopreventive agent. Cancer chemoprevention is targeted at the initiation, promotion, and progression stages of carcinogenesis and requires long-term exposure to non-toxic nutrients, food supplements or pharmacological agents with the aim to prevent the development of malignancies. Xanthohumol and 8-prenylnaringenin were found to inhibit the metabolic activation of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a procarcinogen found in cooked meats, in the Ames Salmonella mutagenicity test and in an assay for binding of IQ metabolites to DNA at low micromolar concentrations (Miranda et al., 2000c). These authors demonstrated that the inhibition of the metabolic activation of this cooked-meat procarcinogen was most likely due to inhibition of cytochrome P450 enzymes, in particular Cyp1A2. They concluded that dietary intake of prenylflavonoids through normal beer consumption would not be sufficient, unfortunately, to achieve plasma concentrations that could inhibit IQ activation in vivo (e.g., 1 μM). Xanthohumol, isoxanthohumol, 8-prenylnaringenin, and nine other prenylflavonoids from hops were shown to strongly inhibit the cDNA-expressed human cytochrome P450

enzymes, Cyp1A1, Cyp1B1, and Cyp1A2, but not Cyp2E1 and Cyp3A4 at low micromolar concentrations (Henderson et al., 2000). In a detailed study by Gerhauser et al. (2002), xanthohumol and isoxanthohumol were also identified as potent inhibitors of Cyp1A activity in vitro with IC_{50} values in the sub-micromolar range. These cytochrome P450s form a group of enzymes that mediate the metabolic activation of many chemical carcinogens, and the inhibitory effects of hop prenylflavonoids on cytochrome P450s may offer an explanation for the reported inhibitory effects of beer on mutagenesis and DNA adduct formation induced by carcinogens (Arimoto-Kobayashi et al., 1999).

Detoxification of carcinogens by Phase 2 enzymes represents another target for cancer chemoprevention. Phase 2 enzymes mediate the conjugation of (P450-activated) xenobiotics including carcinogens to endogenous ligands, such as glutathione, glucuronic acid, acetate, and sulfate, to facilitate excretion. Thus, inhibition of cytochrome P450 enzymes (Phase 1 enzymes) together with induction of Phase 2 enzymes should result in enhanced protection against carcinogenesis in its early stages. NAD(P)H:quinone reductase is a Phase 2 enzyme that is involved in the detoxification of quinones by reductive conversion into hydroquinones, which are suitable substrates for enzymatic conjugation. Xanthohumol and six other prenylated chalcones induced quinone reductase in cultured mouse hepatoma Hepa 1c1c7 cells; the concentrations needed to double quinone reductase activity was in the range 2.1–10.1 μM with xanthogalenol being the most potent inducer (Miranda et al., 2000a). Similar results were obtained for xanthohumol and isoxanthohumol by Gerhauser et al. (2002). Xanthohumol was characterized as a monofunctional inducer, that is, it selectively induces quinone reductase without simultaneously causing transcriptional activation of the Phase 1 enzyme, Cyp1A1 (Miranda et al., 2000a; Gerhauser et al., 2002). Thus, xanthohumol could have beneficial effects on detoxifying carcinogens by inhibition of Phase 1 enzymes and induction of Phase 2 enzymes.

Uncontrolled proliferation of tumor cells has been associated with inflammation and increased production of hormone-like mediators such as prostaglandins. Food constituents that can interfere with proliferation mechanisms are of great interest as cancer chemopreventive agents due to their long-term exposure. Antiproliferative and cytotoxic effects of xanthohumol and five other prenylated hop flavonoids were tested in breast cancer (MCF-7), colon cancer (HT-29), and ovarian cancer (A-2780) cells in vitro (Miranda et al., 1999). Xanthohumol inhibited the proliferation of MCF-7 and A-2780 cells in a dose-dependent manner with IC_{50} values of 13 and 0.52 μM, respectively, after two days of treatment. Gerhauser et al. (2002) showed that xanthohumol can be an effective anti-inflammatory agent by inhibition of endogenous prostaglandin synthesis through inhibition of cyclooxygenase (constitutive COX-1 and inducible COX-2) enzymes with IC_{50} values of 17 and 42 μM, respectively. Prostaglandins are also known to initiate formation of new blood vessels (angiogenesis), an important event in tumor growth. The effect of 8-prenylnaringenin on angiogenesis was studied by Pepper et al. (2004), who demonstrated that 8-prenylnaringenin inhibits angiogenesis in an in vitro model in which endothelial cells can be induced to invade a three-dimensional collagen gel and form capillary-like tubes. Dependent on the hormone stimulus, IC_{50} values between 3 and 10 μM were calculated for the inhibitory effect of 8-prenylnaringenin in this assay. Unfortunately, xanthohumol was not tested by Pepper and co-workers. Zhao et al. (2003) have demonstrated that xanthohumol inhibits the production of nitric oxide (NO) by suppressing the expression of inducible nitric oxide synthase (iNOS). This finding may be relevant to angiogenesis because excessive and prolonged NO generation promotes the production of vascular endothelial growth factor (VEGF), a known inducer of angiogenesis.

6.1. Cancer-related therapeutic application of xanthohumol and 8-prenylnaringenin: will a dream come true?

From the exciting in vitro findings summarized in the preceding paragraphs, we cautiously conclude that xanthohumol and other hop prenylflavonoids have potential as cancer chemopreventive agents by interfering with a variety of cellular mechanisms at low micromolar concentrations: (1) inhibition of metabolic activation of procarcinogens, (2) induction of carcinogen-detoxifying enzymes, and (3) inhibition of tumor growth by inhibiting inflammatory signals and angiogenesis. General cytotoxicity is an important issue in cancer chemoprevention because of the long-term exposure: Miranda et al. (1999) demonstrated that xanthohumol shows no toxicity to liver cells nor does it inhibit mitochondrial respiration and uncouple oxidative phosphorylation in isolated rat liver mitochondria at 10 μM or lower. These levels are unlikely to be achieved by consumption of beer and no cytotoxic effects are to be expected from moderate beer consumption due to the presence of prenylflavonoids. The potential health-maintaining effects of xanthohumol as a 'broad-spectrum' cancer chemopreventive agent have led to the development of experimental beers with high levels of xanthohumol (ca. 5 mg/L) (Biendl et al., 2001). Pharmacokinetic data for xanthohumol, 8-prenylnaringenin, and their metabolites are urgently needed in order to be able to evaluate effect and side effect of 'formulations' containing xanthohumol or 8-prenylnaringenin. Thus, the answer to the above question is: 'maybe, but it is far too early to tell for sure'.

7. Antioxidant activities

Flavonoids represent the largest group of plant polyphenols. Their dietary intake through fruits and vegetables has been associated with a lower incidence of cardiovascular disease (Hertog et al., 1993). This and many other studies suggest that dietary flavonoids may have beneficial effects on human health and disease prevention, which is primarily attributed to their antioxidant properties. While commonly occurring flavonols have received much attention as natural antioxidants, only a few studies have reported on the antioxidant effects of prenylated flavonoids, probably because of their low dietary intake compared to flavonols and anthocyanins. In a study conducted by Miranda et al. (2000b), prenylated chalcones from hops protected low density lipoprotein (LDL) from Cu^{2+}-induced oxidation in vitro. Of the 12 tested prenylated chalcones, xanthohumol and desmethylxanthohumol were the most effective antioxidants. At 5 μM, these compounds decreased conjugated diene formation, a measure of lipid peroxidation, by more than 70% after 5 h of incubation compared to the vehicle control (LDL and Cu^{2+}). In these in vitro assays, xanthohumol was more effective than α-tocopherol and the isoflavone genistein but less effective than the flavonol quercetin, while isoxanthohumol and the non-prenylated flavonoids, naringenin and chalconarigenin, showed no antioxidant activity (Miranda et al., 2000b). Similarly, the inhibitory effect of hop flavonoids on liver microsomal lipid peroxidation, induced by Fe^{2+}-ascorbate, Fe^{3+}-ADP/NADPH, or *tert*-butyl hydroperoxide, was greater for monoprenylated chalcones than for their non-prenylated analogs. However, an increase in the number of prenyl substituents caused the antioxidant activity to decrease in lipid peroxidation assays (Rodriguez et al., 2001).

Scavenging of reactive oxygen species (hydroxyl, peroxyl, and superoxide anion radicals) represents one of the mechanisms by which flavonoids exert their antioxidant activities. Free radicals can initiate reactions that modify polyunsaturated lipids, proteins and nucleic acids, which have been associated with the early stages of atherosclerosis and carcinogenesis, and with the development of neurodegenerative diseases (Halliwell and Gutteridge, 1999). Scavenging of reactive oxygen species by xanthohumol and isoxanthohumol was studied by Gerhauser et al. (2002), who found that xanthohumol was 8.9- and 2.9-fold more potent that the reference compound trolox at a concentration of 1 μM with respect to scavenging of hydroxyl and peroxyl radicals in the ORAC assay. Xanthohumol was also shown to scavenge superoxide anion radicals, generated by xanthine oxidase, without directly inhibiting xanthine oxidase activity. Furthermore, xanthohumol inhibited superoxide anion radical formation by 12-*O*-tetradecanoylphorbol-13-acetate stimulation of differentiated HL-60 human promyelocytic leukemia cells with and IC_{50} of 2.6 μM. Isoxanthohumol was inactive in both superoxide scavenging assays (Gerhauser et al., 2002).

8. Estrogenic activity

The estrogenic effects of hops have been recognized for decades (Zenisek and Bednar, 1960). Haensel and Schulz (1988) suspected that their desmethylxanthohumol was identical with an earlier reported 'pro-estrogen' from hops, from which the 'hop-estrogens', 6-, and 8-prenylnaringenin, are formed by spontaneous or base-catalyzed cyclization. The active estrogenic principle remained unclear, however, until bioassay-guided fractionation of hop extracts led to the isolation 8-prenylnaringenin as a potent phytoestrogen (Milligan et al., 1999). 8-Prenylnaringenin appeared more active than the well-recognized phytoestrogens, genistein and daidzein, in a yeast screen bearing the human estrogen receptor and a receptor binding assay using rat uterine cytosol. Although 8-prenylnaringenin is a much weaker estrogen than 17β-estradiol (<1%), 8-prenylnaringenin is the most potent phytoestrogen known to date. Interestingly, the 5-*O*-methyl derivative of 8-prenylnaringenin, isoxanthohumol, has no estrogenic activity. Weak estrogenic activities were observed for 6-prenylnaringenin, 8-geranylnaringenin, and 6,8-diprenylnaringenin, while other hop prenylflavonoids, including xanthohumol and xanthogalenol showed very weak or no estrogenic activities in the yeast screen (Milligan et al., 2000).

The (2*R*) and (2*S*)-enantiomers of 8-prenylnaringenin showed similar estrogenic activities in the yeast screen and in estrogen-responsive human endometrial Ishikawa Var-I cells (Milligan et al., 2002). Schaefer et al. (2003) found that both 8-prenylnaringenin enantiomers show high affinity and strong selectivity for the α form of the estrogen receptor (ERα) in a competition assay using recombinant human ERα and ERβ from cytosolic SF9-cell extracts. 2*S* (−)-8-prenylnaringenin exhibited an overall higher affinity for both receptors than the 2*R* enantiomer. In comparison with genistein, 8-prenylnaringenin was found to be a 100 times more potent ERα agonist but a much weaker agonist of ERβ in the estradiol-competition assay for receptor binding. These authors claim that 8-prenylnaringenin is the strongest plant-derived ERα receptor agonist identified to date. In an in vivo study, the estrogenic potency of 8-prenylnaringenin was about 20,000-fold lower compared to 17β-estradiol using uterine and vagina growth assays (Schaefer et al., 2003). These distinctive estrogenic properties of 8-prenylnaringenin will foster new research on 8-prenylnaringenin's potential as a selective estrogen receptor modulator ('SERM') for treatment of perimenopausal problems (hot flashes) and for prevention

of osteoporosis in postmenopausal women. In this context it is interesting to note that xanthohumol and humulone, not 8-prenylnaringenin, were identified in bio-assay guided fractionation of hop extract as surprisingly strong inhibitors of bone resorption with IC_{50} values of about 1 μM and 6 nM, respectively (Tobe et al., 1997). Postmenopausal osteoporosis has been associated with estrogen deficiency leading to an imbalance between bone formation and bone resorption. Xanthohumol shows no appreciable estrogenic activity in in vitro assays and therefore its capability to inhibit bone resorption seems difficult to explain through estrogenic pathways.

8.1. Is 8-prenylnaringenin a phytochemical or an artifact?

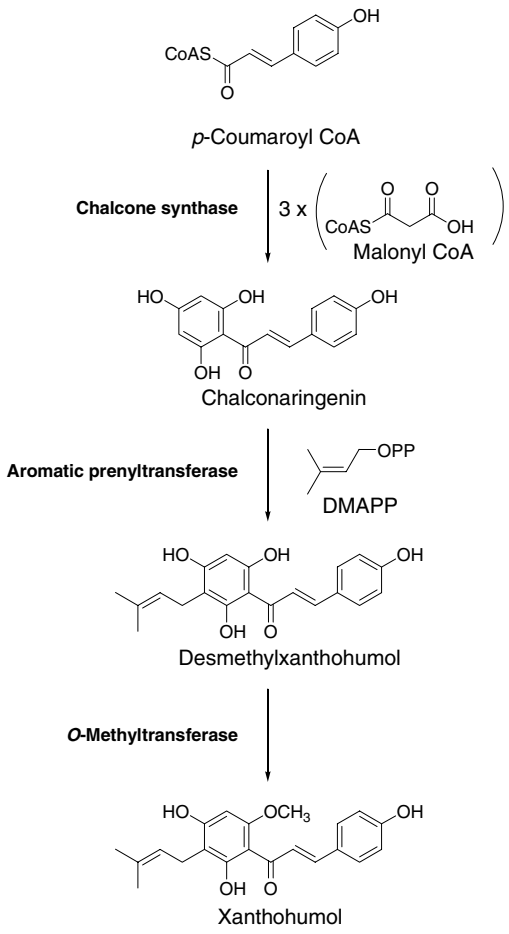
There has been a discussion as to whether 8-prenylnaringenin is produced by the hop plant or that the prenylated naringenins of hops are artifacts of the corresponding chalcones. We are of the opinion that most if not all of the 8-prenylnaringenin content of hop extracts and beer originate from 8-prenylnaringenin's precursor, desmethylxanthohumol, by spontaneous or heat-induced isomerization in aqueous solvents during the extraction process, and therefore 8-prenylnaringenin should be present in hop extracts as a mixture of enantiomers. This assumption is based on the observation that cold alcoholic extracts of fresh hop cones contain much more desmethylxanthohumol than 6- or 8-prenylnaringenin, the latter being barely detectable in such extracts (Haensel and Schulz, 1988; Stevens et al., 2000). Furthermore, we have determined that desmethylxanthohumol has a half-life of less than 5 min in the brew kettle, where it is largely converted into 6- and 8-prenylnaringenin (Stevens et al., 1999a).

8.2. Dietary intake of 8-prenylnaringenin and health effects

Beer is the most important dietary source of 8-prenylnaringenin. Most hopped beers contain less than 100 μg of 8-prenylnaringenin/L; the highest concentration of 8-prenylnaringenin measured in a microbrew beer was 240 μg/L (Table 2). In mice treated with 8-prenylnaringenin, the minimal concentration to produce a significant increase in vaginal mitosis was 100 μg/mL drinking water. No effect on uterine growth was observed at this concentration (Milligan et al., 2002), roughly 400–1000-fold higher than the 8-prenylnaringenin concentration in beer. It seems therefore safe to assume that human exposure to phytoestrogens through beer consumption causes no detrimental health effects. A risk assessment is more difficult to make for consumption of herbal supplements containing hops due to lack of data on prenylflavonoid contents of most commercial products and intake. Further research on the estrogenic activity of 8-prenylnaringenin in different tissues is required to determine whether high doses from supplements have the potential to increase the risk of breast or uterine cancer. The safety aspect should be a real concern because any claimed beneficial effect, such as reduction of hot flashes and breast enhancement, has never been proven for such herbal supplements in properly controlled clinical trials to our knowledge.

9. Biosynthesis of prenylflavonoids

Although prenylflavonoids like xanthohumol have a restricted distribution in nature, flavonoids are ubiquitous in higher plants and the biosynthetic pathway leading to these compounds is well characterized at both genetic and enzymatic levels (Forkmann and Martens, 2001; Winkel-Shirley, 2002). The first step in flavonoid biosynthesis is the condensation of *p*-coumaroyl CoA with three molecules of malonyl CoA to give chalconaringenin (also called naringenin chalcone), a reaction catalyzed by chalcone synthase (CHS, E.C. 2.3.1.74) (Scheme 1). A chalcone synthase gene, *chs*-H1, was cloned from hops and found to be part of a multigene family consisting of at least six members (Matousek et al., 2002a,b). The biosynthesis of many flavonoids proceeds via the enzymatic conversion of this tetrahydroxychalcone to a flavanone, (2*S*)-naringenin, through the action of chalcone isomerase (CHI, E.C. 5.5.1.6) (Jez et al., 2000). This isomerization also occurs non-enzymatically to give an enantiomeric mixture of (2*R*)- and (2*S*)-naringenin. In the case of xanthohumol, however, subsequent prenylation and methylation of the A-ring appears to inhibit flavanone formation and diverts flavonoid biosynthesis into a specialized metabolic branch. Prenylation of the A ring with dimethylallyl diphosphate (DMAPP) yields desmethylxanthohumol which is subsequently methylated at the 6′-hydroxyl group to form xanthohumol. Although the order of these two reactions is not clear, the detection of desmethylxanthohumol in hops (Stevens et al., 1997) suggests that prenylation occurs before methylation and our biosynthetic scheme (Scheme 1) reflects this. On the other hand, methylation slows down the rate of isomerization to the flavanone (due to chelation of the remaining free hydroxyl group with the nearby keto functionality), so perhaps this reaction occurs before prenylation. As we note earlier, desmethylxanthohumol isomerizes to 6- and 8-prenylnaringenin during brewing, and it is unclear why this does not occur in vivo. The cellular environment where prenylflavonoids are formed could be hydrophobic, which may help prevent isomerization.



Scheme 1. Biosynthesis of xanthohumol.

The prenyltransferase catalyzing desmethylxanthohumol formation has not been characterized; indeed there is limited information about aromatic prenyltransferases in plants. The few that have been cloned include homogentisate phytyltransferase, which is involved in tocopherol biosynthesis (Collakova and DellaPenna, 2001), and a geranyltransferase participating in the formation of shikonin, a naphthoquinone in *Lithospermum erythrorhizon* (Yazaki et al., 2002). From hops, Zuurbier et al. (1998) detected aromatic prenyltransferase enzymes catalyzing the sequential prenylation of polyketide intermediates in humulone and cohumulone biosynthesis. As well, an aromatic geranyltransferase that functions in the biosynthesis of cannabinoids, terpenophenolic metabolites formed by similar route as prenylflavonoids, has been biochemically characterized in *Cannabis sativa* L. (Fellermeier and Zenk, 1998). It is interesting to note that the *Humulus* and *Cannabis* aromatic prenyltransferases were found to be soluble enzymes. In many ways, the biosynthesis of prenylflavonoids parallels that of hop bitter acids such as humulone. In an isotope labeling study of humulone biosynthesis, Goese et al. (1999) found that the C5 prenyl groups, and hence the DMAPP from which they derive, was formed via the plastidic deoxyxylulose phosphate (DXP) pathway. We speculate that the C5 prenyl side-chain of xanthohumol also has its origin in the DXP pathway.

The O-methylation step, whether it proceeds via desmethylxanthohumol or chalconaringenin, has also not been elucidated in hops. A chalcone *O*-methyltransferase (ChOMT) that catalyzes a similar reaction in *Medicago sativa* L. has been cloned (Maxwell et al., 1993) and structurally characterized (Zubieta et al., 2001). ChOMT methylates the 2′-OH of the isoliquiritigen, using *S*-adenosyl-L-methionine (SAM) as a methyl donor, to produce 4,4′-dihydroxy-2′-methoxy chalcone. This compound functions as a nodulation inducer in *Rhizobium* spp. (Maxwell et al., 1993). A cDNA sequence encoding a putative isoliquiritigenin/licodione 2′-*O*-methyltransferase involved in the formation of the retrochalcone echinatin in licorice (*Glycyrhiza echinata* L.) is present in GenBank (D88742). This sequence and that of *M. sativa* ChOMT grouped together in two phylogenetic analyses of plant *O*-methyltransferases (Ibrahim et al., 1998; Joshi and Chiang, 1998). It seems likely that the hops desmethylxanthohumol *O*-methyltransferase or chalconaringenin *O*-methyltransferase has similar properties to other plant chalcone OMTs.

10. Chemical ecology of prenylflavonoids

Prenylflavonoids and bitter acids accumulate in lupulin glands that cover bracts (also called scales) of female hop cones (Fig. 1). The biosynthetic pathways leading to these compounds are highly active in such glands with both *chs*_H1 and valerophenone synthase, the polyketide synthase involved in bitter acid formation (i.e., humulone), expressed at high levels (Okada and Ito, 2001; Matousek et al., 2002a). Since hops also contain kaempferol and quercetin glycosides (Sägesser and Deinzer, 1996), the enzymes that function in xanthohumol biosynthesis divert flavonoid biosynthesis into the prenylflavonoid pathway only in lupulin glands. From an ecological perspective, terpenophenolics such as xanthohumol and humulone may have evolved to defend against insects that feed on the female cones and the seeds that later develop within them. Little is known about the antifeedant or insecticidal activities of prenylflavonoids but hop bitter acids show activity against insects (Jones et al., 1996; Powell et al., 1997). It is interesting to note that other terpenophenolics such as the psychoactive cannabinoids are also localized in glandular trichomes on *Cannabis* female flower bracts, and that prenylated phloroglucinols (e.g., hypercalin A) are found in levels of up to 20% by dry weight in the ovarian

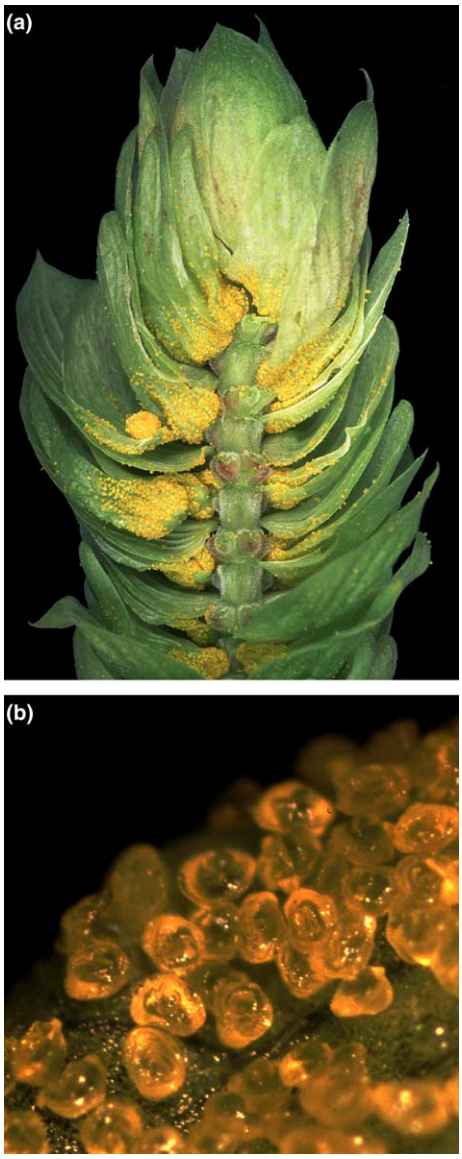


Fig. 1. Lupulin glands on female inflorescences (cones) of *H. lupulus*. Bitter acids and prenylflavonoids are present in yellow glandular trichomes, termed lupulin glands, the cover the bracts of hops cones (a). (b) Magnified view of lupulin glands on a bract.

wall of *Hypercium calycinum* L. (Guttiferae). In all these instances it appears that terpenophenolics are produced to defend female reproductive structures against insects or other herbivores.

11. Opportunities for metabolic engineering of prenylflavonoids

Beer is the alcoholic beverage of choice in many parts of the world and may represent a vehicle for increasing the consumption of natural products with antioxidant and other health-promoting properties. We should note, however, that the health benefits of moderate amounts of beer and other alcoholic beverages must be balanced against the health problems associated with alcohol abuse. Based on the health-promoting properties of xanthohumol, increasing the concentration of this compound in beer might be beneficial. One way to achieve higher prenylflavonoid levels would be to add it to the brewing process (Biendl et al., 2001), although this would require extraction and purification of large amounts of these chemicals. Another route would be to increase prenylflavonoid levels in hops through breeding or metabolic engineering of their biosynthesis. Genetic transformation of hops has been reported (Horlemann et al., 2003). Considering the attitudes of some consumers to GMO foods, and the "purity" of beer in the eyes of its drinkers, traditional or marker-assisted breeding approaches may lead to a more marketable product at present.

Metabolic engineering of flavonoid biosynthesis has been achieved through the overexpression of specific enzymes in the pathway or of regulatory factors controlling flavonoid biosynthesis. Muir et al. (2001) reported a 78-fold increase in flavonol levels in tomato fruit peel by overexpressing petunia CHI. Simultaneous expression of several flavonol biosynthetic enzymes in tomato fruit also led to increased flavonoid levels (Colliver et al., 2002). In a different approach to increasing flavonoids in tomatoes, ectopic expression of maize transcription factors that control flavonoid biosynthesis gave a 20-fold increase in total fruit flavonols (Bovy et al., 2002). Although these examples of metabolic engineering involve flavonols, increased prenylflavonoid levels in hops might be obtained through similar means. Higher amounts of prenylflavonoids could possibly be obtained through the knockout of the pathway leading to bitter acids (e.g., valerophenone synthase) which would make more polyketide and terpenoid precursors available for prenylflavonoid formation. The resulting high xanthohumol/low bitter acid hops could be used together with bitter acid containing material for brewing. An alternative route to engineering hops with both higher prenylflavonoid and bitter acid levels would be to increase the number of lupulin glands on hops cones. Such engineering of secretory tissues has been suggested for other species (McCaskill and Croteau, 1999) but would require much information about the complex developmental pathways that give rise to glandular trichomes.

Metabolic engineering of prenylflavonoid biosynthesis could also be directed at developing hops varieties that contain higher amounts of 8-prenylnaringenin for pharmaceutical use. Currently large-scale synthesis of 8-prenylnaringenin is not cost-effective. Based on the attenuation of chalcone isomerization by the presence of a 6′-methoxy group, knockout of the *O*-methyl-

transferase responsible for this metabolic step would prevent xanthohumol formation and lead to the accumulation of desmethylxanthohumol. The latter compound, in the presence of CHI, could then isomerize to 6- or 8-prenylnaringenin in vivo. Knockout of the desmethylxanthohumol *O*-methyltransferase or chalconaringenin *O*-methyltransferase could be accomplished via transformation of hops with RNAi silencing, or through targeted non-transgenic approaches such as TILLING (targeting induced local lesions in genomes) (Henikoff and Comai, 2003).

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