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## REVIEWS

# Citrus Limonoids: Analysis, Bioactivity, and Biomedical Prospects

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Limonoids are a prominent group of secondary metabolites in citrus fruit. The bitter character of some compounds in this group has historically compromised the quality of citrus fruit and juice. Detecting bitter limonoids in citrus, understanding their origins, and developing methods for their removal from citrus juices have provided the basis for citrus limonoid research. Evaluation of the biological activity of citrus limonoids has indicated the potential of these compounds to improve human health as anticancer, cholesterol-lowering, and antiviral agents. This review chronicles the evolution of citrus limonoid research from defining their participation in citrus bitterness to their potential utilization as important contributors to improving human health and well-being.

**KEYWORDS: Limonoids; citrus; bitterness; human health; review**

## INTRODUCTION

Citrus fruits are recognized as an important component of the human diet, providing a variety of constituents important to human nutrition, including vitamin C, folic acid, potassium, flavonoids, pectin, and dietary fiber. Citrus also contains significant amounts of highly oxygenated triterpenoid compounds (limonoids), particularly in underutilized byproducts of citrus juice production.

Limonoids occur naturally only in plant species of the Rutaceae and Meliaceae plant families. A short review of research on the limonoids, including citrus limonoids, in these two plant families has recently been reported (*1*), and an overview of citrus limonoid research has been presented as a portion of an ACS symposium (*2*).

This review chronicles the evolution of citrus limonoid research from the discovery of the involvement of these compounds in the development of delayed bitterness in citrus juices to the potential of citrus limonoids as bioactive compounds important to human health and well-being. The review will not consider the biosynthetic origins of limonoids in citrus since the biosynthesis of these compounds has been adequately reviewed (*3, 4*).

## CITRUS LIMONOIDS AND DELAYED BITTERNESS

The juices of many varieties of citrus are susceptible to the development of delayed bitterness attributable to changes in limonoid content. The susceptibility of citrus to bitterness development was a major factor contributing to freeze losses up to $90 million in California in 1992 and 2006. The potential for bitterness also restricts certain commercial citrus varieties (e.g., navel orange) to marketing primarily as table fruit rather than as a source of orange juice.

The general association of delayed bitterness in citrus juices with a triterpenoid (limonoid) was first established in the 19th century (*5*). The correlation of delayed bitterness with a specific bitter limonoid component did not occur until 1938, when limonin (**1**, **Figure 1**) was isolated from navel orange juice (*6*). Limonin was subsequently established to be the primary specific source of delayed bitterness in navel orange juice in 1949 (*7*). The chemical structure of limonin remained unknown until 1968, when it was elucidated by a combination of chemical and physical (X-ray crystallography) methods (*8, 9*).

Delayed bitterness develops in the juice of citrus fruit suffering physical damage or damage from a field freeze event. Physical disruption of the juice sacs in the citrus fruit initiates the biochemical transformation of a tasteless limonoid aglycone precursor (e.g., limonate A-ring lactone (LARL, **2**)) to a bitter limonoid aglycone (limonin (**1**)). This biochemical transformation is catalyzed by the enzyme limonin D-ring lactone hydrolase at pH 6.5 or lower (*10*). The development of bitter limonin is dependent on the LARL pool (*11, 12*). In early season navel oranges, limonin has been observed to continue to be formed from the LARL pool up to 48 h after a freeze event (*13*). The presence of limonin in excess of 6 ppm has been established as an objectionable level of bitterness in citrus and processed citrus products (*14*).

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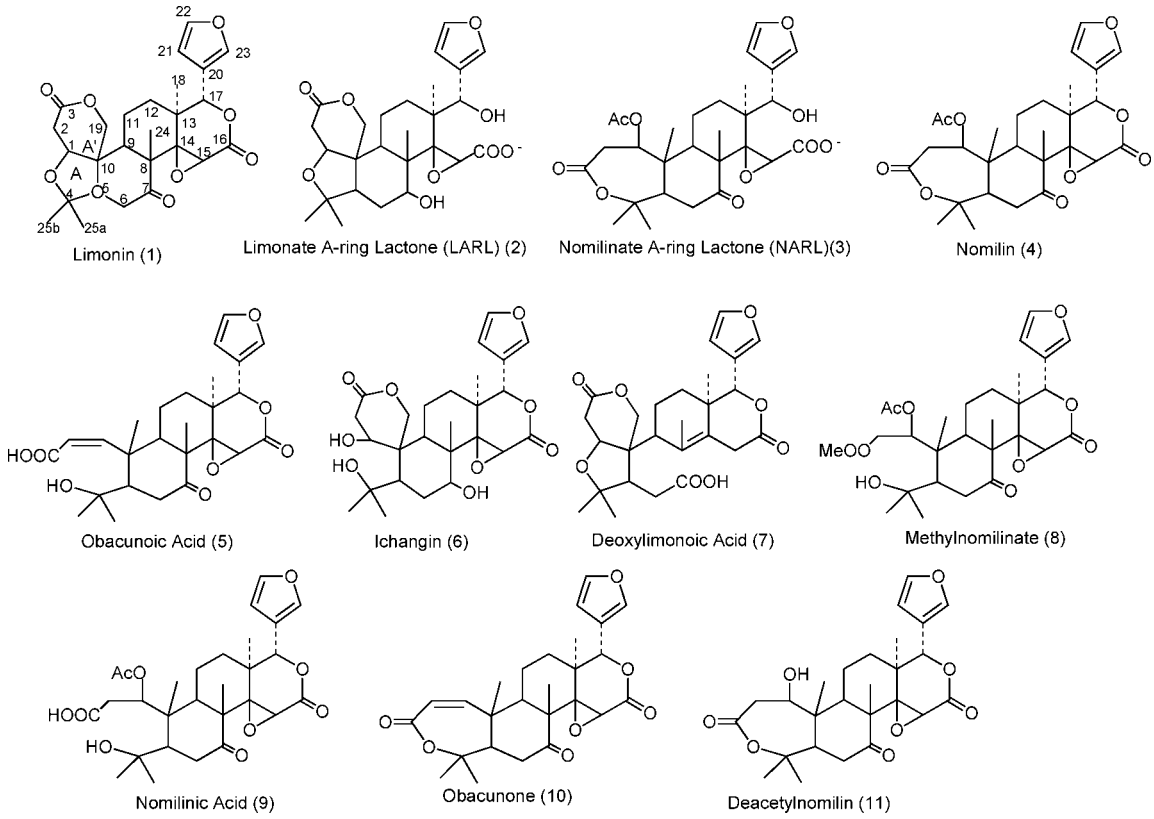


**Figure 1.** Citrus limonoid aglycones.

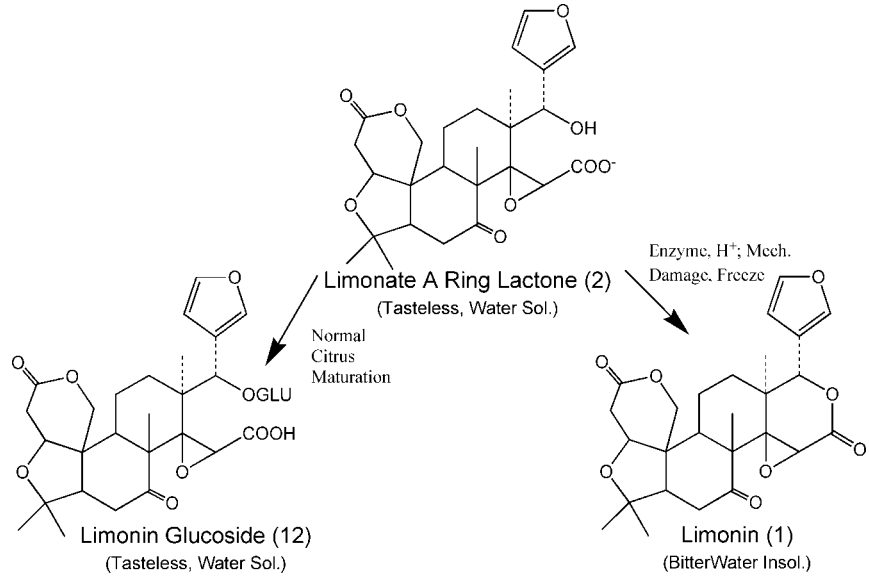


**Figure 2.** Delayed bitterness and natural debittering of limonoids in citrus.

Bitter limonoid aglycones are only present in citrus juice if the enzymatic biochemical transformation of a tasteless open A-ring limonoid aglycone precursor has been initiated. Until recently, the ability to monitor the amount of these precursors in citrus juice has been achieved by measuring bitter limonoid amounts after conversion of the tasteless precursor (*11*, *12*). The recent isolation of the pure limonoid aglycone precursors LARL (**2**) and nomilinate A-ring lactone (NARL) (**3**) (*15*) has now led to a liquid chromatography–mass spectrometry (LC–MS) analytical method (*16*) that can directly quantify the open A-ring limonoid aglycone precursors in citrus samples.

Citrus seeds constitute the only natural repository of citrus limonoid aglycones. The seeds can contain limonoid aglycones in amounts up to 1% of their fresh weight (*17*). Citrus limonoid aglycones have been isolated from the seeds of *Citrus* and related hybrids by solvent extraction (*18*, *19*), buffer extraction (*17*, *20*), and supercritical fluid (*21*, *22*) methods, and almost 40 limonoid aglycones purified by fractional crystallization (*17*, *23*) or chromatography have been characterized (*18*, *23*–*32*). The nuclear magnetic resonance (NMR) spectra of 22 limonoid aglycones have recently been summarized (*33*).

Limonin (**1**) is consistently the most abundant limonoid aglycone found in citrus seeds and is one of only six (limonin, nomilin (**4**), obacunoic acid (**5**), ichangin (**6**), deoxylimonoic acid (**7**), and nomilinic acid (**9**)) limonoid aglycones that have been identified to be inherently bitter (*34*). The correlation of citrus limonoid structural character and perceived bitterness has

established the presence of a closed D-ring, a $C_{14}$–$C_{15}$ epoxide, a C-7 keto group, and an acetyl ester group at C-1 in a seven-membered A-ring as requirements for bitterness (*34*).

The need to monitor delayed bitterness in citrus juices has spawned a wide variety of analytical methods to quantify limonoid aglycones. These citrus limonoids can be semiquantitatively monitored by thin-layer chromatography (*35, 36*) utilizing Ehrlich's reagent (*18*) as a specific detection method. Quantitative methods for analysis of limonoid aglycones primarily involve high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detection. These HPLC–UV methods require high-purity solvents with minimal UV end absorption to accommodate the 207 nm UV absorbance maxima of the limonoids. Numerous reverse-phase HPLC–UV methods utilizing C-18 bonded silica absorption media and acetonitrile/water and acetonitrile/aqueous acid mobile phases have been developed and are described in earlier reviews (*23, 37*). These HPLC–UV methods have limonoid aglycone detection limits near 1 ng. Two normal-phase HPLC–UV methods incorporating binary (*38*) and tertiary (*39*) organic solvent systems on silica HPLC columns have also been reported.

Radioimmunoassay (*40*) and enzyme-linked immunoassay (*41*) methods have also been developed to detect limonoids at very low concentrations. Unfortunately, these methods are reported to be compromised by cross-reactivity between limonin and other nonbitter limonoids present in citrus (*42, 43*).

The development of benchtop mass spectrometers coupled to HPLC systems (LC–MS) has generated new, highly sensitive methodology to detect citrus limonoids in citrus samples. Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) LC–MS utilizing reverse-phase HPLC systems have been shown to be capable of detecting and quantifying bitter citrus limonoids at nanogram levels (*44*). Accuracy in the quantification of limonoids by LC–MS requires incorporation of internal standards or rigorous sample bracketing by external standards to accommodate the inherently dynamic nature of LC–MS.

The identification of specific limonoid aglycones in citrus extracts, chromatographically separated by HPLC–UV methods, relies upon comparisons with the chromatographic properties of standards (*38*). The lack of availability of limonoid aglycone standards, particularly for minor limonoid aglycones, severely inhibits the application of HPLC–UV methods to identify and quantify limonoid aglycones in citrus tissues.

APCI LC–MS, electron ionization (EI) LC–MS, and APCI tandem mass spectrometry (MS/MS) have been applied to pure limonoid aglycones to obtain fragmentation patterns that can be used as an identification tool for unknown limonoid aglycones in citrus extracts. Ammonia-enhanced negative ion and acetic acid-enhanced positive ion APCI LC–MS of four limonoid aglycones (limonin (**1**), nomilin (**4**), obacunone (**10**), and deacetylnomilin (**11**)) established specific ion–adduct patterns for the four limonoids (*45*). Negative ion mode collision dissociation APCI MS/MS also revealed specific fragmentation patterns for the four limonoids (*45*). Direct insertion EI mass spectrometry for the same four limonoids generated mass spectrometric fingerprints for the limonoids without molecular ions (*46*). EI fragmentation pathways were proposed for each of the limonoid aglycones. Seventeen limonoid aglycone standards analyzed by particle beam EI LC–MS (*47*) and positive ion APCI LC–MS provided fragmentation and pseudomolecular ion information that can be correlated with similarly obtained LC–MS data of unknown limonoid aglycones to assist in structural characterization.

## NATURAL DEBITTERING—LIMONOID GLYCOSIDES

The difference in susceptibility of different varieties of citrus to the development of bitterness (e.g., navel vs Valencia) and the intraseasonal change in susceptibility of particular bitterness susceptible varieties (i.e., navel) (*13*) suggested that citrus possesses a natural mechanism to alleviate bitterness. The discovery of tasteless limonoid glycosides in the juice of citrus in the late 1980s (*48*), coupled with the earlier observation that the concentration of the bitter limonin precursor LARL (**2**) decreased as citrus matured (*49*), led to the elucidation of the mechanism of natural debittering in citrus (**Figure 2**). UDP-D-glucose:limonoid glucosyltransferase, purified from the albedo of Frost navel orange, was established as the enzyme catalyzing the glucosylation of LARL (*50*).

Intraseason analysis of the levels of LARL (**2**) and the glucoside of limonin (limonin glucoside (**12**)) in navel and Valencia fruit established that limonin glucoside concentration increased with fruit maturity while LARL concentration decreased (*11, 12*). These studies also confirmed that Valencia orange more efficiently converts LARL to the tasteless limonin glucoside than navel orange, thereby explaining the higher susceptibility of the navel orange to the development of delayed bitterness.

More than 20 limonoid glycosides (all as glucosides) have been isolated and characterized from the tissues of members of the genus *Citrus* and related genera in the plant family Rutaceae (*51*). These tasteless, water-soluble limonoid glucosides occur in citrus fruit tissues, juice, and seeds in high concentrations. The concentration of these compounds in citrus juice ranges from ~150 to 300 ppm (*52, 53*), while the limonoid glucoside content of citrus peel and flesh solids is ~500 ppm (*11, 54*). Citrus seeds contain limonoid glucosides in about 1% of their dry weight (*55*).

Limonin glucoside (**12**, **Figure 3**) is the predominant limonoid glucoside present in citrus juices, occurring in about twice the amount of the other limonoid glucosides combined (*53*). In citrus seeds, nomilin glucoside (**13**) occurs in higher concentration than limonin glucoside (*55*).

Limonin glucoside (**12**), nomilin glucoside (**13**), and other limonoid glucosides have been obtained chromatographically from polar extracts of defatted citrus seeds (*23*) and structurally characterized (*48, 55–61*). The characterized limonoid glucosides are mono-glucosylated mono- or dicarboxylic acid derivatives of A- and D-ring limonoid aglycones. Characterization of the limonoid glucosides has been dependent upon nuclear magnetic resonance spectroscopy (*33*). Only two of the limonoid glucosides (limonin glucoside and deacetylnomilinic acid glucoside (**14**)) have been crystallized.

**Limonoid Glucoside Analysis.** The discovery of limonoid glucosides in citrus initiated a need for analytical methods for their detection and quantification. The water-soluble nature of these compounds requires minimal sample preparation for liquid samples (juice and juice process washes) prior to analysis. Aqueous samples or extracts are commonly filtered, applied to C-18 or styrene divinyl benzene solid-phase extraction (SPE) cartridges, washed with water, eluted with a polar solvent, diluted to standard volume, and analyzed (*52–54*). Limonoid glucoside analytical samples obtained from solid citrus juice processing samples (peel, juice processing solids) require extraction of the citrus solid with water or methanol/water mixtures prior to SPE concentration (*54*). Citrus seeds contain considerable amounts of fats and oils and must be defatted with a nonpolar solvent (hexane) prior to solvent extraction with acetone and methanol to obtain a limonoid glucoside-enriched extract (*23, 62*). The limonoid glucoside-enriched seed extract

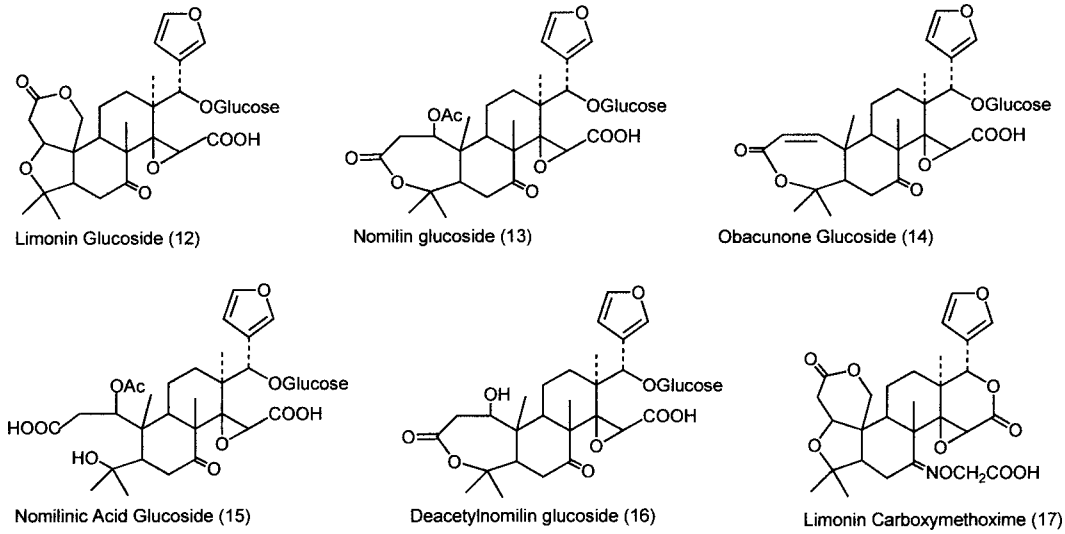


**Figure 3.** Limonoid glucosides and synthetic limonin carboxymethoxime.

is reconstituted in water and prepared utilizing the SPE concentration method, or it can be back-extracted with methylene chloride to remove analysis-interfering components prior to analysis.

Limonoid glucosides can be detected on thin-layer chromatography (TLC) plates with Ehrlich's reagent as with limonoid aglycones. A single solvent system (ethyl acetate/methyl ethyl ketone/formic acid (88%)/water (5:3:1:1)) has been used to achieve chromatographic separation of these compounds by TLC (*52, 62*). The limonoid glucosides can be evaluated semiquantitatively through a comparison of the intensity of the response of individual limonoid glucosides to Ehrlich's reagent with the intensity of a standard limonoid glucoside run on the same TLC plate.

Limonoid glucoside detection and analysis is primarily achieved by reverse-phase HPLC–UV and, more recently, by reverse-phase LC–MS. The reverse-phase HPLC–UV methods developed to quantify limonoid glucosides from citrus sources (*52, 53, 55, 62, 63*) commonly employ C-18 bonded silica stationary media and combinations of acetonitrile or methanol with aqueous acids as eluents. The limonoid glucosides are detected by UV at 210–215 nm and quantified by peak area comparisons (*52, 53*) or comparison with pure limonoid glucosides as external standards (*55, 62, 63*).

Capillary electrophoresis (CE) has recently been applied for the analysis of limonin glucoside (**12**) from citrus seeds, fresh and dried peel, molasses, and finisher pulp (*64*). The CE method utilized UV detection (214 nm) to quantify limonin glucoside with a detection limit of 2 mg/L. Sample quantification utilized pure limonin glucoside as an external standard.

As with the limonoid aglycones, LC–MS offers methodology to detect low levels of limonoid glucosides in citrus samples with minimal solvent-associated complications. LC–MS further provides mass spectrometric data that can assist in the specific identification of citrus limonoids. ESI LC–MS has been applied in positive and negative ion modes to qualitatively detect the protonated and deprotonated molecular ions for limonoid glucosides in citrus peel extracts (*65*) and, in the presence of ammonia, to detect the protonated and deprotonated molecular ion ammonium adducts of limonoid glucosides in extracts of grapefruit seeds (*45*). ESI LC–MS has also been applied in negative ion mode to quantify limonoid glucosides (2 ng detection limit) in extracts of citrus peel, seed, juice, juice-processing solids, and molasses, utilizing carminic acid as an internal standard (*66*).

Application of collision-activated dissociation (CAD) of limonoid glucoside protonated molecular ions in a tandem quadrapole LC–MS (ESI) operated in positive ion mode provided protonated molecular ions for the corresponding limonoid aglycone and additional identifying MS/MS fragments (*45*). CAD has also been applied to deprotonated molecular ions of limonoid glucosides in an ion trap mass spectrometer to acquire characteristic fragmentation patterns for limonoid glucosides obtained from grapefruit seeds (*67*).

## RECOVERY OF CITRUS LIMONOIDS FROM CITRUS-PROCESSING WASTE

Orange juice processing byproducts (peel and flesh solids) contain about 50% of the total limonoid glucosides in whole oranges, about twice the amount of limonoid glucosides in the processed juice from these oranges (*54*). For each ton of whole oranges containing 500 ppm total limonoid glucosides processed to juice, the processing byproducts would contain 1 lb of limonoid glucosides. In 2006–2007, 2.2 million tons of oranges were processed to juice worldwide. The amount of limonoid glucosides available in the processing byproducts of the 2006–2007 juice production would be approximately 1100 tons.

Byproduct solids of citrus juice processing are processed (pressed) to obtain the monoterpene limonene, and the remaining solids are treated with lime to hydrolyze citrus pectins. The resulting pressed liquor is concentrated to molasses. The molasses can contain limonoid glucosides concentrations from 1300 to almost 5000 ppm (*68, 69*). The molasses and the dried pressed solids are commonly utilized as cattle feed. The 1100 tons of limonoid glucosides estimated to occur in orange juice processing byproducts in 2006–2007 is indicative of the citrus limonoid resource available in solid waste byproducts of citrus fruit processing. Additional amounts of limonoid aglycones are available in citrus seeds.

The high concentration of limonoid glucosides (1300–5000 ppm) and the physical character of citrus molasses (water soluble, viscous liquid) have made this material the object of methods development for limonoid glucoside reclamation. Separation methodology targeting the acidic properties, aromatic furan functionality, and water solubility of the limonoid glucosides has been developed (*69*). This methodology utilizes ion-exchange and styrene divinylbenzene (SDVB) resins to separate mixtures of limonoid glucosides from Maillard reaction-derived impurities, interfering phenolic compounds, and sugars. The ion-

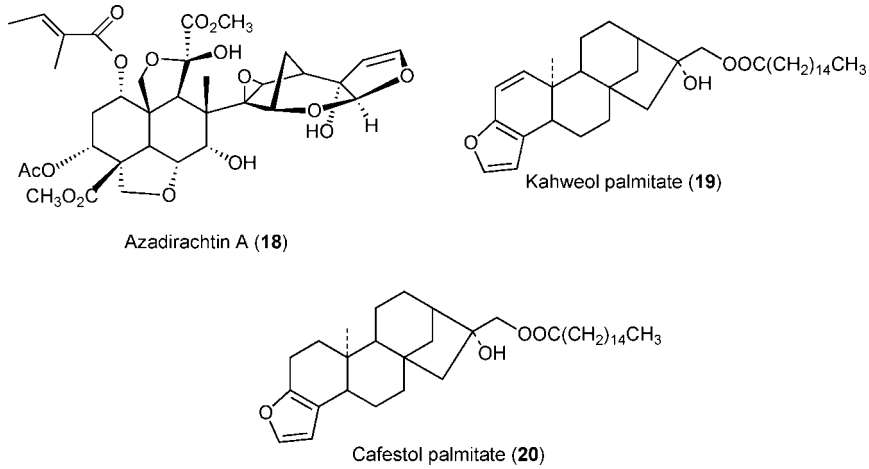


**Figure 4.** Azadiractin and furan-containing antineoplastic agents.

exchange/SDVB absorption resin purification methodology is the basis for an industrial-scale procedure to obtain limonoid glucoside mixtures from citrus molasses (*70*). The ion-exchange/SDVB resin separation methodology has also been coupled with aromatic selective reverse-phase chromatography to isolate individual limonoid glucosides from citrus molasses (*71*). Supercritical fluid extraction methods have also been utilized to obtain mixtures of limonoid glucosides obtained from ion-exchange/SDVB absorption resin isolation (*72*) and directly from citrus molasses (*73*). Citrus molasses is the most attractive source of limonoid glucosides; however, the complex nature of the molasses represents a continuing challenge to its economic utilization.

Citrus limonoids are also obtained from solid waste byproducts of citrus processing. Limonoid aglycones and limonoid glucosides are obtained from citrus seeds by sequential solvent extraction (*18, 19*), and the limonoid aglycones can also be selectively obtained from the seeds by buffer extraction (*17, 20*) and supercritical fluid (*21, 22*) methods. Large-scale amounts of limonoid glucosides (mixture) can be obtained from aqueous extracts of solid citrus-processing waste products by a method (*74*) that utilizes dilute alcohol solutions to selectively elute limonoid glucosides from SDVB absorption resin media. This methodology is currently the only semi-industrial-scale source of citrus limonoids.

## BIOACTIVITY OF CITRUS LIMONOIDS

The correlation of naturally occurring meliacin-type limonoids in the Meliaceae plant family to natural insect resistance provided early evidence of the biological activity of this class of compounds. Azadiractin (**18**, **Figure 4**), a meliacin-type limonoid isolated from the neem tree (*Azadiracta indica*), has become a well-known natural insecticide with efficacy across a broad spectrum of insects (*75*). Many other meliacin-type limonoids have been obtained from *Azadiracta* sp., and several have been shown to display biological activity against human cancer cells *in vivo* (*76*).

The basic structure of the limonoids from *Citrus* sp. and related genera in the Rutaceae differs from that of the meliacin-type limonoids; however, both group of limonoids incorporate a distinctive furan or hydrated furan functionality and epoxide and lactone functional groups. The established biological activity of the meliacin-type limonoids, coupled with the commonality of furan functionality with two chemopreventative compounds found in oil of green coffee beans, kahweol and cafestol (**19** and **20**, **Figure 4**), led to the *in vivo* biological evaluation of citrus limonoids as antineoplastic agents.

**Anticancer Bioassay of Citrus Limonoids (*in Vivo*).** The antineoplastic properties of several citrus limonoids have been studied in mouse (*77–79*) and hamster (*80–82*) model systems. In mice, limonin (**1**) and nomilin (**4**), administered by gavage or included in the diet of animals exposed to benzo[*a*]pyrene, were found to effectively inhibit forestomach and lung tumor growth (*77*). The two limonoids were also shown to be effective in inhibiting 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced two-stage skin carcinogenesis in mice (*78*). In the initial hamster study, in which the oral buccal pouch was treated with DMBA and DMBA plus limonin (**1**), comparison of tumor number, tumor mass, and tumor burden revealed a 20–60% reduction in tumor numbers and size (*80*). A considerably lower reduction of antitumor activity was observed for nomilin (**4**). In the subsequent hamster studies (*81, 82*), the antitumor activity of three limonoid glucosides, limonin glucoside (**12**), nomilin glucoside (**13**), and nomilinic acid glucoside (**15**), was compared with that of controls in the DMBA-treated hamster buccal pouch model system. In these studies, the limonin glucoside-treated hamsters showed a decrease of tumor burden of 55%. Hamsters treated with nomilin glucoside and nomilinic acid glucoside displayed significantly lower antitumor activity. A synthetic derivative of limonin, limonin carboxymethoxime (**17**), evaluated in the hamster study, also showed antitumor activity comparable to that of limonin glucoside. Evaluation of deoxylimonoic acid (**7**) in the hamster model showed the synthetically derived compound to have no activity, suggesting the importance of the C-14,15 expoxide and an intact B-ring in the biological activity of the limonoids.

The capacity of two limonoid aglycones, limonin (**1**) and obacunone (**10**), to inhibit azomethane (AOM)-induced colon tumorgenesis has been evaluated in a rat model system (*83–85*). In these studies, the limonoid aglycones were included as dietary supplements to rats exposed to AOM, and the modifying effects of the limonoids were measured by changes in aberrant crypt foci (ACF). Study results revealed significant reductions in ACF in the colon of the animals and that inclusion of limonoids in the diet at the AOM tumor-induced initiation phase significantly reduced the incidence of colonic adenocarcinoma. Corroborative evidence of the chemopreventative properties of limonoids toward colon cancer has been presented in a study of the ability of limonin, grapefruit pulp, and the flavonoid naringin to mitigate AOM-induced colon carcinogenesis in rats (*86*).

**Anticancer Bioassay of Citrus Limonoids (*in Vitro*).** The antitumor activity observed for limonoids in *in vivo* animal studies has led to the biological activity assessments of these

compounds in *in vitro* human cancer cell studies. Sixteen limonoids (aglycones, glucosides and synthetic analogues) and a mixture of limonoid glucosides have been surveyed for their effect on the proliferation and viability of MDA-MB-435 estrogen receptor-negative (positive) human breast cancer cells in culture (*87*). In this study, deacetylnomilin (**11**) and an undefined mixture of limonoid glucosides were found to be the best inhibitors of estrogen receptor-negative cell growth ($IC_{50}$ < 0.1 $\mu$g/mL). Nomilin glucoside (**13**) was found to be the most effective limonoid glucoside, with activity about one-tenth that of deacetylnomilin. Nomilin (**4**) was the most effective limonoid aglycone, with activity about twice that of its glucoside. All other limonoid aglycones and glucosides had activity from 10 to 400 times lower than that of deacetylnomilin. Limonin methoxime (**17**), a synthetic limonoid analogue, was the most potent inhibitor of estrogen receptor-negative cell growth, with activity about 3 times that of deacetylnomilin.

Deacetylnomilin (**11**) and nomilin glucoside (**13**) were also found be the most effective inhibitors of estrogen receptor-positive breast cancer cells. The activity of deacetylnomilin against the estrogen-positive cells was more than 10 times its activity against the estrogen-negative cells, while the nomilin glucoside activity was similar for both cell lines. The limonoid glucoside mixture's inhibitory activity was also somewhat comparable between the cell lines. The most effective synthetic limonoid analogue for controlling the estrogen receptor-positive cells was methyl nomilinate (**8**), with activity twice that observed for deacetylnomilin (**11**). All other limonoids and synthetic limonoid derivatives were at least 400 times less active than deacetylnomilin.

The cancer chemopreventative drug tamoxifen was included in the study as a reference standard for comparison. A comparison of the activity with that of the limonoids revealed that deacetylnomilin (**11**) was $>10^3$ times more potent than tamoxifen against estrogen receptor-negative cancer cells and >10 times more effective than tamoxifen in inhibiting estrogen receptor-positive breast cancer cells. A subsequent study examining the inhibitory properties of obacunone glucoside (**14**), nomilinic acid glucoside (**15**), limonin (**1**), nomilin (**4**), and a mixture of limonoid glucosides confirmed that limonoids significantly inhibit MCF-7 human breast cancer cell growth (*88*). The limonoids were not significantly effective against five other cancer cell lines included in the study.

Limonoid aglycones and glucosides have also been evaluated as chemopreventatives against SH-SY5Y neuroblastoma and Caco-colon carcinoma cells *in vitro* (*89–91*). In these studies, the cell lines were exposed to 1–50 $\mu$M concentrations of the aglycones limonin (**1**), nomilin (**4**), obacunone (**10**), and deacetylnomilin (**11**) and the corresponding limonoid glucosides (**12**, **13**, **14**, **16**). While all of the limonoids displayed inhibitory activity, the glucosides were more effective than the aglycones at lower concentrations (5 $\mu$M), with clear evidence of induced apoptosis. Furthermore, evidence of toxic selectivity was demonstrated in a comparison of the effect of the limonoids against neuroblastoma and colon carcinoma cell vs Chinese hamster ovary cells (*91*). In this study, it was determined that the Chinese hamster ovary cells were not compromised by concentrations of limonoids that induced apoptosis and suppressed the growth of both cancer cell lines *in vitro*.

**Anticancer Activity of Citrus Limonoids—Mode of Action.** The specific mode of action associated with the antineoplastic activity observed for citrus limonoids in *in vivo* animal bioassays has not been determined. However, the furan structural commonality of the citrus limonoids with the compounds in green coffee bean oil (kahweol (**19**) and cafestol (**20**)), found to induce phase II enzyme glutathione S-transferase (GST) activity *in vivo* (*92*), prompted the investigation of the ability of citrus limonoids to induce GST activity in mice (*93*). The mouse study encompassed eight limonoids, including limonin (**1**) and nomilin (**4**), and established that nomilin increased GST activity 3 times above that of controls in the small intestinal mucosa of test mice. Limonin was not as effective. Results of a separate study that utilized a technique to measure the GST activity of oral epithelial cells in hamster (*94*) confirmed the GST activity of kahweol and cafestol; however, evaluation of limonin glucoside (**12**) revealed no significant GST activity. A study (*95*) examining the effect of limonin and nomilin on phase I P450 and phase II GST activity in rats confirmed that the two limonoids induced GST in a dose-dependent manner in rat liver. In that study, nomilin was also observed to induce GST activity in the small intestine of the test animals. More recently, an examination of the capacity of citrus limonoids and flavonoids to induce GST activity in mice (*96*) confirmed nomilin to be highly effective for GST induction, while limonin was significantly less effective. As previously observed, the limonoid glucosides lacked activity.

**Citrus Limonoids as Antioxidants.** Recognition of the ability of many natural products in foods to intervene in free radical reactions associated with tumorgenesis in mammalian systems has led to the evaluation of the antioxidant capacity of citrus limonoid and several citrus limonoid-containing extracts (*91, 97, 98*). These investigations report antioxidant capacities for specific limonoids over a broad range from undetectable to greater than that of ascorbic acid. A more recent investigation (*99*) of the antioxidant capacities of purified samples of limonin, nomilin, and limonin glucoside, following standardized guidelines for antioxidant capacity measurement (*100, 101*), determined that the three compounds tested showed no antioxidant activity. The study attributed previous discrepancies in antioxidant capacity measurements for citrus limonoids to impurities in limonoid test samples.

The lack of antioxidant capacity of citrus limonoids is consistent with their chemical structure (lack of stabilizing conjugated unsaturation and electron delocalization potential). There is currently no evidence that the mode of action of these compounds in combating tumorgenesis involves antioxidant action.

**Hypocholesterolemic Activity of Citrus Limonoids.** Evidence of the potential of citrus limonoids to possess hypocholesterolemic properties was first obtained in a study of the effect of orange and grapefruit juice consumption on cholesterol metabolism in rabbits (*102, 103*). These studies monitored cholesterol metabolism in cholesterolemic rabbits maintained on diets including double-strength orange and grapefruit juices as a replacement for drinking water. The significant lowering of LDL cholesterol (43% and 32% for orange and grapefruit juice, respectively) compared to controls observed in the study was attributed to secondary metabolites in the juice. A second study (*104*), investigating the hypocholesterolemic effect of dietary orange juice in 15 human subjects with elevated plasma cholesterol levels, revealed that a 750 mL dose of orange juice daily increased HDL levels by 21% and triglyceride levels by 30%. The LDL/HDL ratio was found to decrease by 16% in the test subjects. These results, coupled with the results of the rabbit study, expanded the anecdotal evidence that secondary metabolites, including flavonoids and limonoids, could function endogenously to lower LDL cholesterol. These two classes of

natural products have subsequently been evaluated for their capacity to impact cholesterol metabolism in *in vitro* test systems.

An *in vitro* test system employing cultured HepG2 human liver cells has been utilized to examine the ability of 14 limonoid aglycones, glucosides, and a limonoid glucoside mixture to lower the structural protein of LDL cholesterol (apoB) and to correspondingly quantify their cholesterol-lowering potential (*105*). The study showed that limonin (**1**) reduced apoB production in the liver cells by >70%, while other limonoid aglycones showed lower effectiveness. In contrast, the limonoid glucosides, including limonin glucoside (**12**), showed no effectiveness in reducing apoB in the cultured cells. Comparison of the action of the limonoids on apoB production with prior observations for flavonoids established that limonoids reduced apoB production by a distinctly different mechanism.

Since citrus juices contain high levels of limonoid glucosides (150–300 ppm) compared to the limonoid aglycones (<5 ppm), the ineffectiveness of limonoid glucosides to lower cholesterol in the *in vitro* test system, compared to the observed changes in HDL and triglycerides in humans consuming daily rations of orange juice, seems anomalous. A bioavailability study (*106*) of citrus limonoids in humans provides information that addresses this anomaly as it applies to the participation of these compounds in lowering cholesterol levels and/or acting as antineoplastic agents in humans. In this study, three different concentrations of pure limonin glucoside (in 250 mL of water) were fed to 16 human subjects. Analysis of plasma generated from periodic blood draws over 24 h revealed low levels (nanomolar) of limonin. The level of limonin was found to peak within the first 6 h after ingestion and, in contrast to flavonoid bioavailability findings, to persist, at reducing levels, for 24 h. These data substantiate that limonin, derived from limonin glucoside, can become bioavailable to act as a hypocholesterolemic agent in humans.

Confirmation of the bioavailability of limonoid aglycone in humans fed a limonoid glucoside describes an ideal delivery system of a water-insoluble bioactive compound (limonoid aglycone) from an abundant, water-soluble derivative (limonoid glucoside). The hypocholesterolemic effects observed for citrus juices in the *in vivo* human studies vs *in vitro* human cancer cell lines confirm the importance of this concept. Conversely, there is a paucity of data to support this natural delivery system in the case of limonoid aglycones or limonoid glucosides administered to induce GST activity and correspondingly initiate antineoplastic activity. While early *in vivo* studies (*93, 95*) of the GST induction activity of limonoid aglycones are confirmatory, limonoid glucosides were not included. More recent *in vivo* results (*96*) included limonin glucoside but reported that it did not display GST induction activity. *In vitro* study results of hamster oral epithelial cells (*94*) reported no GST activity for limonoid glucosides, but an *in vivo* study on oral cancers in hamsters (*82*) showed limonin glucoside to reduce oral cancer character at levels comparable to those achieved with the limonoid aglycones tested. Clearly, more *in vivo* animal studies are needed to determine if the administration of water-soluble limonoid glucosides can provide GST-inducing limonoid aglycones to act as effective antineoplastic agents in the manner observed for their apparent role as hypocholesterolemic agents.

**Antiviral Activity of Citrus Limonoids.** Limonin (**1**) and nomilin (**4**) have also been evaluated for their capacity to act as antiretroviral agents (*107*). The effect of the two compounds on the growth of human immunodeficiency virus-1 (HIV-1) was examined in a culture of human peripheral blood mononuclear cells (PBMC) and on monocytes/macrophages (M/M). Both compounds were found to inhibit viral replication in the $EC_{50}$ = 50–60 $\mu$M range. The limonoids were effective at inhibiting HIV-1 replication in infected M/M at concentrations ranging from 20 to 60 $\mu$M. The mechanism of action was attributed to inhibition of *in vitro* HIV-1 protease activity.

**Insecticidal Activity of Citrus Limonoids.** The observed insecticidal properties of azadiractin (**18**) and structurally related meliacin-type limonoids led to the evaluation of the insecticidal properties of prominent limonoids in *Citrus* (Rutaceae). An early bioassay of limonin (**1**), nomilin (**4**), and obacunone (**10**) activity relative to that of corn earworm and fall army worm control established limonin (**1**) to effectively disrupt larvae development (*108*). Subsequent evaluation of the insecticidal properties of individual citrus limonoids toward other agricultural insect pests confirmed the earlier observations, and more recent research with limonin and its derivatives has shown that citrus limonoids possess significant antifeeding and oviposition suppression properties against the Colorado potato beetle (*109*). Field test results provide evidence that the insecticidal citrus limonoids can function as an important component of an integrated pest management program to control Colorado potato beetle populations.

## CITRUS LIMONOIDS AND HUMAN HEALTH

The *in vivo* and *in vitro* evaluations of citrus limonoid biological activity, coupled with the high concentration of these compounds in citrus fruit and juices, suggest their inherent participation in the contribution of citrus to human health. Additionally, three *in vivo* studies (*87, 110, 111*) have corroborated epidemiological evidence that citrus limonoids are not toxic to humans. The studies established that animals consuming a diet containing up to 3% limonoid aglycones or limonoid glucosides suffered no toxic consequences. The animal toxicity data translates to a human consumption of 130 glasses of orange juice per day per week for a proportionate toxic effect.

The experimental evidence of the bioactivity of limonoids in citrus, coupled with their lack of mammalian toxicity, desirable physical properties, and bioavailability to humans, supports the continued consideration of the utilization of these compounds to improve human health. In particular, the physical characteristics (water solubility, tastelessness) of limonoid glucosides make these compounds ideal candidates as nutraceuticals or as constituents in functional foods.

Realization of the potential of citrus limonoids to benefit human health will require the accumulation of more conclusive evidence of the scope of their bioactive benefits to humans through human trials. Human study test data validating the importance of these compounds to humans will drive the expansion of nutrition-related research and rationalize the refinement of industrial-scale production methodology. Initially, human testing of these compounds should concentrate on non-cancer-related health issues, i.e., cholesterol lowering. The successful correlation of the citrus limonoids with improving human health will ultimately define the economic value of these compounds and correspondingly serve to overcome the technological barriers for the production-scale reclamation methods necessary to economically reclaim the compounds from citrus-processing byproducts.

The economic realization of citrus limonoids as nutraceuticals or as important components of functional foods benefiting human health should also expand interest in the chemistry and biochemistry of this group of compounds. Specific correlation of the chemistry or biochemistry of these compounds in relation to their mode of action will enhance their potential realization

as antineoplastic therapeutics. More information about the specific limonoid structure properties in relationship to their observed bioactivity will provide a basis for selective chemical or biochemical methods to enhance antitumor activity. The development of lower molecular weight, water-soluble derivatives (i.e., salts) of limonoid aglycones could enhance delivery concentration. Research exploring the potential of defined synergism involving the citrus limonoids, citrus flavanoids, and other citrus secondary metabolites could yield nutraceutical mixtures with higher efficacy in lowering cholesterol. Any or all of these research efforts will serve to establish and maximize the contribution of bioactive citrus secondary metabolites to benefiting human health and well-being.

## LITERATURE CITED


(1) Roy, A.; Saraf, S. Limonoids: Overview of significant bioactive triterpenes distributed in plants kingdom. *Biol. Pharm. Bull.* **2006**, *29*, 191–201.

(2) Hasegawa, S.; Berhow, M. A.; Manners, G. D. Citrus limonoid research: An overview. In *Citrus limonoids: Functional chemicals in agriculture and foods*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 1–7.

(3) Hasegawa, S.; Miyake, M. Biochemistry and biological function of citrus limonoids. *Food Rev. Int.* **1996**, *12*, 413–435.

(4) Hasegawa, S. Biochemistry of limonoids in *Citrus*. In *Citrus limonoids: Functional chemicals in agriculture and foods*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 9–30.

(5) Bernay, S. Limonin. *Liebig's Ann.* **1841**, *40*, 317.

(6) Higby, R. H. The bitter constituents of navel and Valencia oranges. *J. Am. Chem. Soc.* **1938**, *60*, 3013–3018.

(7) Emerson, O. H. The bitter principle of navel oranges. *Food Technol. (Chicago, IL)* **1949**, *3*, 243–250.

(8) Arnott, S.; Davie, A. W.; Robertson, J. M.; Sim, G. A.; Watson, D. G. The structure of limonin. *Experientia* **1960**, *16*, 49–51.

(9) Arnott, S.; Davie, A. W.; Robertson, J. M.; Sim, G. A.; Watson, D. G. The structure of limonin: X-ray analysis of epilimonol iodoacetate. *J. Chem. Soc.* **1961**, 4183–4200.

(10) Maier, V. P.; Hasegawa, S.; Hera, E. Limonin D-ring lactone hydrolase, a new limonin derivative in citrus. *Phytochemistry* **1969**, *8*, 243–248.

(11) Fong, C. H.; Hasegawa, S.; Coggins, C. W., Jr.; Atkins, D. R.; Miyake, M. Contents of limonoids and limonin 17-B-D-glucopyranoside in fruit tissue of valencia orange during fruit growth and maturation. *J. Agric. Food Chem.* **1992**, *40*, 1178–1181.

(12) Hasegawa, S.; Ou, P.; Fong Chi, H.; Herman, Z.; Coggins, C. W., Jr.; Atkins, D. R. Changes in the limonate-A-ring lactone and limonin 17-B-D-glucopyranoside content of navel oranges during fruit growth and maturation. *J. Agric. Food Chem.* **1991**, *39*, 262–265.

(13) Manners, G. D. *The feasability of colorimetric analysis for the determination of freeze impacted citrus quality*; California Citrus Research Board: Visalia, CA, 2005; p 1.

(14) Guadagni, D. G.; Maier, V. P.; Turnbaugh, J. G. Effects of some citrus constituents on taste thresholds for limonin and naringin bitterness. *J. Sci. Food Agric.* **1973**, *24*, 1277–1288.

(15) Zukas, A. A.; Breksa, A. P., III; Manners, G. D. Isolation and characterization of limonoate and nomilinoate A-ring lactones. *Phytochemistry* **2004**, *65*, 2705–2709.

(16) Breksa, A. P., III; Zukas, A. A.; Manners, G. D. Determination of limonoate and nomilinoate A-ring lactones in citrus juices by liquid chromatography–electrospray ionization mass spectrometry. *J. Chromatogr. A* **2005**, *1064*, 187–191.

(17) Hasegawa, S.; Bennett, R. D.; Verdon, C. P. Limonoids in citrus seeds: Origin and relative concentration. *J. Agric. Food Chem.* **1980**, *28*, 922–925.

(18) Dryer, D. L. Limonoid bitter principles. In *Progress in the chemistry of organic natural products*; Zechmeister, L., Ed.; Springer: New York, 1965; Vol. 26, pp 191–244.

(19) Emerson, O. H. The bitter principles of citrus fruit. I. Isolation of nomilin, a new bitter principle from the seeds of oranges and lemons. *J. Am. Chem. Soc.* **1948**, *70*, 545–549.

(20) Miyake, M.; Ayano, S.; Ozaki, Y.; Maeda, H.; Ifuku, Y.; Hasegawa, S. Extraction of neutral limonoids from citrus seeds. *Nippon Nogeikagaku Kaishi* **1991**, *65*, 987–992.

(21) Ayano, S.; Ozaki, Y.; Inaba, N.; Miyake, M.; Haeda, H.; Ifuku, Y.; Hasegawa, S. Extraction of limonoids and limonoid glucosides with supercritical carbon dioxide. *Nippon Shokuhin Kogyo Gakkaishi* **1992**, *39*, 684–689.

(22) Patil, B. S.; Yu, J.; Dandekar, D. V.; Toledo, R. T.; Singh, R. K. Pike, L. M. Citrus bioactive limonoids and flavonoids extraction by supercritical fluids. In *Potential health benefits of citrus*; Patil, B. S., Turner, N. D., Miller, E. D., Brodbelt, J. S., Eds.; American Chemical Society: Washington, DC, 2006; Vol. 936, pp 18–33.

(23) Herman, Z.; Fong, C. H.; Hasegawa, S. Analysis of limonoids in citrus seeds. In *Modern methods of plant analysis: Seed analysis*; Linskens, H. F., Jackson, J. F., Eds.; Springer-Verlag: Berlin, 1992; pp 361–375.

(24) Lee, S.-Y.; Morita, H.; Takeya, K.; Itokawa, H.; Fukaya, H. Limonoids from Citrus nippokereana. *Nat. Med. (Tokyo)* **1999**, *53*, 255–258.

(25) Nakagawa, H.; Duan, H.; Takaishi, Y. Limonoids from Citrus sudachi. *Chem. Pharm. Bull. (Tokyo)* **2001**, *49*, 649–651.

(26) Bennett, R. D. Acidic limonoids of citrus seeds. *Phytochemistry* **1971**, *10*, 3065–3068.

(27) Bennett, R. D.; Hasegawa, S. Isolimonoic acid, a new citrus limonoid. *Phytochemistry* **1980**, *19*, 2417–2419.

(28) Bennett, R. D.; Hasegawa, S. Limonoids in Calamondin seeds. *Phytochemistry* **1981**, *37*, 17–24.

(29) Bennett, R. D.; Hasegawa, S.; Wong, R. Y. Limonoids from *Atalantia zeylanica*. *Phytochemistry* **1994**, *36*, 163–166.

(30) Bennett, R. D.; Herman, Z.; Hasegawa, S. Ichangensin: A new citrus limonoid. *Phytochemistry* **1988**, *27*, 1543–1545.

(31) Dryer, D. L. Citrus bitter principals. VI. Ichangin. *Tetrahedron* **1966**, 2279–2281.

(32) Teng, J.; Yang, X.-W. A new limonoid from the fruits of *Evodia ruteacarpa* (Juss.) Benth. *Pharmazie* **2006**, *61*, 1038–1040.

(33) Manners, G. D.; Hasegawa, S.; Bennett, R. D.; Wong, R. Y. LC–MS and NMR Techniques for the analysis and characterization of *Citrus* limonoids. In *Citrus Limonoids: Functional chemicals in agriculture and food*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 40–59.

(34) Hasegawa, S.; Miyake, M.; Ozaki, Y.; Berhow, M. A. Limonin: A non-volitile bitter principle in citrus juice. In *The contribution of low- and non-volitile materials to the flavor of foods*; Pickenhagen, W., Ho, C. T., Spanier, A. W., Eds.; Allured Publishing: Carol Stream, IL, 1996; pp 137–145.

(35) Hasegawa, S. Analysis of limonoids by thin-layer chromatography. In *Citrus limonoids: Functional chemicals in agriculture and foods*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 31–39.

(36) Maier, V. P.; Grant, E.R. Specific thin-layer chromatography assay of limonin, a citrus bitter principle. *J. Agric. Food Chem.* **1970**, *18*, 250–252.

(37) Hasegawa, S.; Berhow, M. A.; Fong Chi, H. Analysis of bitter principles in *Citrus*. In *Fruit analysis*; Linskens, H. F., Jackson, J. F., Eds.; Springer-Verlag: Berlin, 1996; Vol. 18, pp 59–80.

(38) Manners, G. D.; Hasegawa, S. A new normal phase liquid chromatographic method for the analysis of limonoids in *Citrus*. *Phytochem. Anal.* **1999**, *10*, 76–81.

(39) Rouseff, R. L.; Fisher, J. F. Determination of limonin and related limonoids in citrus juices by high performance liquid chromatography. *Anal. Chem.* **1980**, *52*, 1228–1233.

(40) Mansell, R. L.; Weiler, E. W. Radioimmunoassay for the determination of limonin in citrus. *Phytochemistry* **1980**, *19*, 1403–1407.

(41) Jorudan, P. S.; Manselolo, R. L.; Weiler, E. W. Competitive solid phase enzyme-linked immunoassay for the quantification of limonin in citrus. *Anal. Biochem.* **1984**, *138*, 19–24.
(42) Rouseff, R. L.; Mansell, R. L. Comparison of high performance liquid chromatographic and radioimmunoassay limonin values from commercial grapefruit juice. *Proc. Fla. State Hortic. Soc.* **1982**, *95*, 249–252.
(43) Widmer, W. W.; Rouseff, R. L. Quantitative analysis of limonin in grapefruit juice using an enzyme-linked immunoassay: collaborative study. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 513.
(44) Manners, G. D.; Breksa, A. P., III; Schoch, T. K.; Hidalgo, M. B. Analysis of bitter limonoids in citrus juices by atmospheric pressure chemical ionization and electrospray ionization liquid chromatography–mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 3709–3714.
(45) Tian, Q.; Schwartz, S. J. Mass spectrometry and tandem mass spectrometry of citrus limonoids. *Anal. Chem.* **2003**, *75*, 5451–5460.
(46) Tian, Q.; Li, D.; Barbacci, D.; Schwartz, S. J.; Patil, B. S. Electron ionization mass spectrometry of citrus limonoids. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2517–2522.
(47) Manners, G. D.; Breksa, A. P., III. Identifying citrus limonoid aglycones by HPLC-EI/MS and HPLC-APCI/MS techniques. *Phytochem. Anal.* **2004**, *15*, 372–381.
(48) Hasegawa, S.; Bennett, R. D.; Herman, Z.; Fong Chi, H.; Ou, P. Limonoid Glucosides in Citrus. *Phytochemistry* **1989**, *28*, 1717–1720.
(49) Maier, V. P.; Hasegawa, S.; Bennett, R. D.; Echols, L. C. Limonin and limonoids. Chemistry, biochemistry and juice bitterness. In *Citrus nutrition and quality*; Nagy, S., Attaway, J. A., Eds.; American Chemical Society: Washington, DC, 1980; Vol. 143, pp 63–82.
(50) Hasegawa, S.; Suhayda, C. G.; Hsu, W.-J.; Robertson, G. H. Purification of limonoid glucosyltransferase from navel orange albedo tissue. *Phytochemistry* **1997**, *46*, 33–37.
(51) Hasegawa, S.; Fong Chi, H.; Herman, Z.; Miyake, M. Glucosides of limonoids. In *Flavor precursors*; Teranish, R., Takeoka, G. R., Matthias, G., Eds.; American Chemical Society: Washington, DC, 1992; Vol. 490, pp 88–97.
(52) Fong, C. H.; Hasegawa, S.; Herman, Z.; Ou, P. Limonoid glucosides in commercial citrus juices. *J. Food Sci.* **1990**, *54*, 1505–1506.
(53) Herman, Z.; Fong Chi, H.; Ou, P.; Hasegawa, S. Limonoid glucosides in orange juice by HPLC. *J. Agric. Food Chem.* **1990**, *38*, 1860–1861.
(54) Ozaki, Y.; Ayano, S.; Inaba, N.; Miyake, M.; Berhow, M. A.; Hasegawa, S. Limonoid glucosides in fruit, juice and processing by-products of Satsuma Mandarin (*Citrus unshiu* Marcov.). *J. Food Sci.* **1995**, *60*, 186–189.
(55) Ozaki, Y.; Fong, C. H.; Herman, Z.; Maeda, H.; Miyake, M.; Ifuku, Y.; Hasegawa, S. Limonoid glucosides in citrus seeds. *Agric. Biol. Chem.* **1991**, *55*, 137–141.
(56) Bennett, R. D.; Hasegawa, S.; Herman, Z. Glucosides of Acidic Limonoids in Citrus. *Phytochemistry* **1989**, *28*, 2777–2781.
(57) Bennett, R. D.; Miyake, M.; Ozaki, Y.; Hasegawa, S. Limonid glucosides in *Citrus aurantium*. *Phytochemistry* **1991**, *30*, 3803–3805.
(58) Miyake, M.; Ozaki, Y.; Ayano, S.; Bennett, R. D.; Hasegawa, S. Limonin glucosides in calamondin seeds. *Phytochemistry* **1992**, *31*, 1044–1046.
(59) Ozaki, Y.; Miyake, M.; Maeda, H.; Ifuku, Y.; Bennett, R. D.; Hasegawa, S. Limonoid glucosides in *Tetradium retaecarpa*. *Phytochemistry* **1991**, *30*, 2365–2367.
(60) Ozaki, Y.; Miyake, M.; Maeda, H.; Ifuku, Y.; Bennett, R.D.; Herman, Z.; Hasegawa, S. Ichangensin glucoside in *Citrus junos*, *Citrus sudachi* and *Citrus sphaerocarpa*. *Phytochemistry* **1991**, *30*, 2659–2661.
(61) Sawabe, A.; Morita, M.; Kiso, T.; Kishine, H.; Ohtsubo, Y.; Minematsu, T.; Matsubara, Y.; Okamoto, T. Isolation and characterization of new limonoid glycosides from *Citrus unshiu* peels. *Carbohydr. Res* **1999**, *315*, 142–147.
(62) Ohta, H.; Fong Chi, H.; Berhow, M. A.; Hasegawa, S. Thin layer and high-performance liquid chromatographic analysis of limonoids and limonoid glucosides in *Citrus* seeds. *J. Chromatogr. A* **1993**, *639*, 295–302.
(63) Tian, Q.; Miller, E. G.; Jayaprakasha, G. K.; Patil, B. S. An improved HPLC method for the analysis of citrus limonoids in culture media. *J. Chromatogr. B* **2006**, *846*, 385–390.
(64) Braddock, R. J.; Bryan, C. R. Extraction parameters and capillary electrophoresis analysis of limonin glucoside and phlorin in citrus byproducts. *J. Agric. Food Chem.* **2001**, *49*, 5982–5988.
(65) Tian, Q.; Ding, X. Screening for limonoid glucosides in *Citrus tangerina* (Tanaka) Tseng by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. A* **2000**, *874*, 13–19.
(66) Schoch, T. K.; Manners, G. D.; Hasegawa, S. Analysis of limonoid glucosides from Citrus by electrospray ionization liquid chromatography–mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 1102–1108.
(67) Raman, G.; Cho, M.; Brodbelt, J. S.; Patil, B. S. Isolation and purification of closely related citrus limonoid glucosides by flash chromatography. *Phytochem. Anal.* **2005**, *16*, 155–160.
(68) Hasegawa, S.; Fong Chi, H.; Miyake, M.; Keithly, J. H. Limonoid glucosides in orange molasses. *J. Food Sci.* **1996**, *61*, 560–561.
(69) Schoch, T. K.; Manners, G. D.; Hasegawa, S. Recovery of limonoid glucosides from citrus molasses. *J. Food Sci.* **2002**, *67*, 3159–3163.
(70) Ifuku, Y.; Maeda, H.; Inaba, N.; Ayano, S.; Ozaki, Y.; Maruyama, K.; Hasegawa, S. Method of manufacturing limonoid glucosides. U.S. Patent 5,734,046, 1998.
(71) Manners, G. D.; Breksa , A. P., III. Manufacture of limonoid compounds. U.S. 20060116509, 2006.
(72) Miyake, M.; Shimoda, M.; Osajima, Y.; Inaba, N.; Ayano, S.; Ozaki, Y.; Hasegawa, S. Extraction and recovery of limonoids with the supercritical carbon dioxide micro-bubble method. In *Citrus Limonoids: Functional chemicals in agriculture and foods*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 96–106.
(73) Yu, J.; Dandekar, D. V.; Toledo, R. T.; Singh, R. K.; Patil, B. S. Supercritical fluid extraction of limonoid glucosides from grapefruit molasses. *J. Agric. Food Chem.* **2006**, *54*, 6041–6045.
(74) Lang, T. R. Method for selectively extracting bioactive components from a raw material extract. AU Pat. 200,187,353, 2006.
(75) Stone, R. A biopesticidal tree begins to blossom. *Science* **1992**, *255*, 1070.
(76) Cui, B.; Chai, H.; Constant, H. L.; Santisuk, T.; Reutrakul, V.; Beecher, C. W. W.; Farnsworth, N. R.; Cordell, G. A.; Pezzuto, J. M.; Kinghorn, A. D. Limonoids from *Azadiracta excelsa*. *Phytochemistry* **1998**, *47*, 1283–1287.
(77) Lam, L. K. T.; Zhang, J.; Hasegawa, S. Inhibition of chemically induced carcinogenesis by citrus limonoids. In *Phytochemicals for cancer prevention*; Schut, H. A. J., Huang, M. T., Osawa, C. T. H., Rosen, R. T., Eds.; American Chemical Society: Washington, DC, 1994; Vol. 546, pp 209–219.
(78) Lam, L. K. T.; Zhang, J.; Hasegawa, S. Citrus limonoid reduction of chemically induced tumorgenesis. *Food Technol.* **1994**, 104–108.
(79) Lam, L. K. T.; Hasegawa, S.; Bergstrom, C.; Lam, S. H.; Kenney, P. Limonin and nomilin inhibitory effects on chemical induced tumorgenesis. In *Citrus Limonoids: Functional chemicals in agriculture and foods*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 185–200.
(80) Miller, E. G.; Fanous, R.; Rivera-Hidalgo, F.; Binnie, W. H.; Hasegawa, S.; Lam, L. K. The effect of citrus limonoids on hamster buccal pouch carcinogenesis. *Carcinogenesis* **1989**, *10*, 1535–1537.
(81) Miller, E. G.; Gonzales-Sanders, A. P.; Couvillon, A. M.; Binnie, W. H.; Hasegawa, S.; Lam, L. K. T. Citrus limonoids as inhibitors of oral carcinogenesis. *Food Technol.* **1994**, 110–114.

(82) Miller, E. G.; Gonzales-Sanders, A. P.; Couvillon, A. M.; Wright, J. M.; Hasegawa, S.; Lam, L. K. T. Inhibition of hamster buccal pouch carcenogenesis by limonin 17-B-D-glucopyranoside. *Nutr. Cancer* **1992**, *17*, 1–7.

(83) Tanaka, T.; Kohno, H.; Tsukio, Y.; Honjo, S.; Tanino, M.; Miyake, M.; Wada, K. Citrus limonoids obacunone and limonin inhibit azoxymethane-induced colon cancer in rats. *Biofactors* **2000**, *13*, 213–218.

(84) Tanaka, T. Kohno, H.; Kawabata, K.; Honjo, S.; Miyake, M.; Wada, K. Citrus limonoids obacunone and limonin inhibit the development of a precursor lesion, aberrant crypt foci, for colon cancer in rats. In *Citrus limonoids: Functional chemicals in agriculture and foods*' Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 145–163.

(85) Tanaka, T.; Maeda, M.; Kohno, H.; Murakami, M.; Kagami, S.; Miyake, M.; Wada, K. Inhibition of azoxymethane-induced colon carcinogenesis in male F-344 rats by the citrus limonoids obacunone and limonin. *Carcinogenesis* **2000**, *22*, 193–198.

(86) Turner, N. D.; Vanamala, J.; Leonardi, T.; Patil, B. S.; Murphy, M. E.; Liu, Y. C.; Wang, N.; Pike, L. M.; Chapkin, R. S.; Lupton, J. R. Comparison of chemoprotection conferred by grapefruit and isolated bioactive compounds against colon cancer. In *Potential Health Benefits of Citrus*; Patil, B. S., Turner, N. D., Miller, E. D., Brodbelt, J. S., Eds.; American Chemical Society: Washington, DC, 2006; Vol. 936, pp 121–129.

(87) Guthrie, N.; Hasegawa, S.; Manners, G. D.; Carroll, K. K.; Berhow, M. A.; Hasegawa, S.; Manners, G. D. Inhibition of human breast cancer cells by citrus limonoids. In *Citrus Limonoids: Functional chemicals in agriculture and foods*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 164–174.

(88) Tian, Q.; Miller, E. G.; Ahmad, H.; Tang, L.; Patil, B. S. Differential inhibition of human cancer cell proliferation by citrus limonoids. *Nutr. Cancer* **2001**, *40*, 180–184.

(89) Poulose, S. M.; Harris, E. D.; Patil, B. S. Cytotoxic and antineoplastic effects of citrus limonoids against human neuroblastoma and colonic aderocarcinoma cells. *FASEB* **2006**, *20*, A11–A12.

(90) Poulose, S. M.; Harris, E. D.; Patil, B. S. Antiproliferative effects of citrus limonoids against human neuroblastoma and colonic adenocarcinoma cells. *Nutr. Cancer* **2006**, *56*, 103–112.

(91) Poulose, S. M.; Harris, E. D.; Patil, B. S. Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. *J. Nutr.* **2005**, *135*, 870–877.

(92) Wattenberg, L. W.; Lam, L. K. T. Protective effects of coffee constituents on carcinogenesis experimental animals. *Banbury Rep.* **1984**, *17*, 137–145.

(93) Lam, L. K. T.; Li, Y.; Hasegawa, S. Effects of citrus limonoids on glutathione S-transterase activity in mice. *J. Agric. Food Chem.* **1989**, *37*, 878–880.

(94) Miller, E. G.; Gonzales-Sanders, A. P.; Couvillon, A. M.; Wright, J. M.; Hasegawa, S.; Lam, L. K. T.; Sunahara, G. I. Inhibition of oral carcinogenesis by green coffee beans and limonoid glucosides. In *Food phytochemicals for cancer prevention I*; Huang, M. T., Osawa, T., Ho, C.-T., Rosen, R. T., Eds.; American Chemical Society: Washington, DC, 1994; Vol. 546, pp 220–229.

(95) Kelly, C.; Jewell, C.; O'Brien, N. M. The effect of dietary supplementation with the citrus limonoids limonin and nomilin on xenobiotic-metabolizing enzymes in the liver and small intestine of the rat. *Nutr. Res.* **2003**, *23*, 681–690.

(96) Ahmad, H.; Li, J.; Polson, M.; Mackie, K.; Quiroga, W.; Patil, B. S. Citrus limonoids and flavanoids: Enhancement of Phase II detoxification enzymes and their potential in chemoprevention. In *Potential Health Benefits of Citrus*; Patil, B. S., Turner, N. D., Miller, E. D., Brodbelt, J. S., Eds.; American Chemical Society: Washington, DC, 2006; Vol. 936, pp 130–143.

(97) Sun, C.; Chen, K.; Chen, Y.; Chen, Q. Contents and antioxidant capacity of limonin and nomilin in different tissues of citrus fruit of four cultivars during fruit growth and maturation. *Food Chem.* **2005**, *93*, 599–605.

(98) Yu, J.; Wang, L. M.; Walzem, R. L.; Miller, E. G.; Pike, L. M.; Patil, B. S. Antioxidant activity of citrus limonoids, flavanoids and coumarins. *J. Agric. Food Chem.* **2006**, *53*, 2009–2014.

(99) Breksa, A. P., III; Manners, G. D. Evaluation of antioxidant capacity of limonin, nomilin and limonin glucoside. *J. Agric. Food Chem.* **2006**, *54*, 3827–3831.

(100) Decker, E. A.; Warner, K.; Richards, M. P.; Shahidi, F. Measuring antioxidant effectiveness in food. *J. Agric. Food Chem.* **2005**, *53*, 4303–4310.

(101) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.

(102) Kurowska, E. M.; Vandenberg, T. A.; Manthey, J. A.; Hasegawa, S.; Manners, G. D. In *Regulation of cholesterol metabolism by citrus juices, flavonoids and limonoids, 9th Congress of the International Society of Citriculture*; International Society of Citriculture: Orlando, FL, 2000; pp 1192–1194.

(103) Kurowska, E. M.; Borradaile, N. M.; Spence, J. D.; Carroll, K. K. Hypocholesterolemic effects of dietary citrus juices in rabbits. *Nutr. Res.* **2000**, *20*, 121–129.

(104) Kurowska, E. M.; Spence, J. D.; Jordan, J.; Wetmore, S.; Freeman, D. J.; Piche, L. A.; Serratore, P. HDL cholesterol raising effect of orange juice in subjects with hypercholesterolemia. *Am. J. Clin. Nutr.* **2000**, *72*, 1095–1100.

(105) Kurowska, E. M.; Banh, C.; Hasegawa, S.; Manners, G. D. Regulation of apo B production by HepG2 cells by citrus limonoids. In *Citrus Limonoids: Functional chemicals in agriculture and food*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 175–184.

(106) Manners, G. D.; Jacob, R. A.; Breksa, A. P., III; Schoch, T. K.; Hasegawa, S. Bioavailability of citrus limonoids in humans. *J. Agric. Food Chem.* **2003**, *51*, 4156–4161.

(107) Battinelli, L.; Mengoni, F.; Lichtner, M.; Mazzanti, G.; Saija, A.; Mastroianni, C. M.; Vullo, V. Effect of limonin and nomilin on HIV-1 replication on infected human mononuclear cells. *Planta Med.* **2003**, *69*, 910–913.

(108) Klocke, J. A.; Kubo, I. Citrus limonoid by-products as insect control agents. *Entomol. Exper. Appl.* **1982**, *32*, 299–301.

(109) Alford, A. R.; Murray, K. D. Prospects for citrus limonids in insect pest management. In *Citrus limonoids: Functional chemicals in agriculture and foods*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; Vol. 758, pp 201–211.

(110) Miller, E. G.; Gibbins, R. P.; Taylor, S. E.; McIntosh, J. E.; Patil, B. S. Long term screening study on the potential toxicity of limonoids. In *Potential health benefits of Citrus* ; Patil, B. S., Turner, N. D., Miller, E. G., Brodbelt, J. S., Eds.; American Chemical Society: Washington, DC, 2006; Vol. 936, pp 82–94.

(111) Miller, E. G.; Record, M. T.; Binnie, W. H.; Hasegawa, S. Limonoid glucosides: Systemic effects on oral carcinogenesis. In *Phytochemicals and Phytopharmaceuticals*; Shahidi, F., Ho, C., Eds.; AOAC Press: Champaign, IL, 2000; pp 95–105.

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