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Identification and biosynthesis of acylphloroglucinols in *Hypericum gentianoides*

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Abstract

Species of the genus *Hypericum* contain a rich array of unusual polyketides, however, only a small proportion of the over 450 *Hypericum* species, other than the popular medicinal supplement St. John's Wort (*H. perforatum*), have even been chemically characterized. *H. gentianoides*, a small annual used medicinally by Cherokee Americans, contains bioactive acylphloroglucinols. Here, we identify acylphloroglucinol constituents of *H. gentianoides* and determine a potential pathway to their synthesis. Liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) and HPLC-UV indicate that the level of accumulation and profile of acylphloroglucinols in *H. gentianoides* vary little seasonally when grown in a greenhouse, but do vary with development and are highly dependent on the accession, highlighting the importance of the selection of plant material for study. We identify the chemical structures of the nine prevalent polyketides, based on LC/ESI-MS and hybrid quadrupole orthogonal time-of-flight mass (Q-TOF) spectrometry; these metabolites include one monomeric phlorisobutyrophenone (PIB) derivative and eight dimeric acylphloroglucinols. Q-TOF spectrometry was used to identify eight additional PIB derivatives that were not detected by LC/ESI-MS. These data lead us to propose that diacylphloroglucinols are synthesized via modification of PIB to yield diverse phloroglucinol and filicinic acids moieties, followed by dimerization of a phloroglucinol and a filicinic acid monomer to yield the observed complement of diacylphloroglucinols. The metabolomics data from *H. gentianoides* are accessible in PMR (<http://www.metnetdb.org/pmr>), a public metabolomics database with analysis software for plants and microbial organisms.

Introduction

Specialized phytochemicals provide complex defense strategies to combat insects, fungi, animals, or abiotic stressors, as well as attractants for pollination or dispersal. Such phytochemicals encompass tremendous structural diversity, from terpene alkaloids as in *Digitalis purpurea* (Chappell 2008), to polyketide alkylamides as in *Echinacea* (Wu et al. 2009), to waxes and scents (Fatland et al. 2002, Klingauf et al. 2005). Often, specialized metabolites are contained in discreet compartments. For example, peltate glands in the young leaves of sweet basil (*Ocimum basilicum*) accumulate monoterpenes (Iijima et al. 2004); 2-carene, α -terpinene, and limonene are concentrated in the glandular trichomes of

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tomato (*Solanum lycopersicum*) (Schilmiller et al. 2009); and the toxic dianthrone hypericin accumulates in nodular glands of *H. perforatum* (Piovan et al. 2004, Košuth et al. 2011).

Hypericum encompasses over 450 species, few of which have been chemically profiled (Crispin and Wurtele 2013), and is the largest genus within Clusiaceae, fossils of which date to the Late Cretaceous (90 million years ago) (Gustafsson et al. 2002). *H. perforatum* (St. John's Wort) is commonly used by humans medicinally (Ernst 2003, Birt et al. 2009). Extracts of *H. perforatum*, when applied to bacterial, viral or animal systems, induce complex, context-dependent changes to cellular and physiological functions. Medicinally "desirable" bioactivities include: anti-viral (Axarlis et al. 1998, Maury et al. 2009), anti-inflammatory (Hammer et al. 2007), anti-depressive (Mennini and Gobbi 2004, Butterweck and Schmidt 2007, Kasper et al. 2008), and anti-bacterial (Franklin et al. 2009, Saddiqe et al. 2010). Furthermore, these compounds have been used to treat obsessive compulsive disorder (Camfield et al. 2011). Potentially negative effects of *H. perforatum* include: cytotoxicity in HaCaT human keratinocytes (Schmitt et al. 2006); photosensitivity in mammals (Traynor et al. 2005, Onoue et al. 2011); decreased serum concentrations of anti-retroviral and anti-cancer pharmaceuticals in humans (De Maat et al. 2001, Caraci et al. 2011); and increased blood pressure and heart rate in humans taking MAOIs (Tachjian et al. 2010).

A single chemical compound in *H. perforatum*, the acylphloroglucinol hyperforin, is believed to be the source of the anti-depressive activity (Mennini and Gobbi 2004, Brenner et al. 2000, Tu et al. 2010, Carpenter 2011). Hyperforin is also toxic to gram-positive bacteria (Reichling et al. 2001, Avato et al. 2004, Joray et al. 2011), has been shown to be a potential anticarcinogen (Schempp et al. 2002) and is an angiogenesis inhibitor (Schempp et al. 2005). Four less studied, acylphloroglucinols, saroaspidin A, saroaspidin B, uliginosin A, and uliginosin B have also been shown to have a variety of biological activities (Ishiguro et al. 1987, Rocha et al. 1995, Stein et al. 2012)

H. gentianoides (Orange grass, Pineweed), is a small annual that grows throughout the central and eastern United States and Canada (USDA Natural Resources Conservation Service <http://plants.usda.gov/java/profile?symbol=HYGE>). Cherokee Native Americans used this plant to cure ailments as mild as nosebleeds and sores, and as severe as fever and venereal diseases (Hamel and Chiltoskey 1975). Interestingly, neither hypericin nor hyperforin have been detected in this species (Crockett et al. 2005, Hillwig et al. 2008). Overall, *H. gentianoides* has been characterized by a few studies of its biological activities (Hillwig et al. 2008, Huang et al. 2011) and in relation to its geographical distribution (Bliss et al. 2002). Despite lacking hypericin or hyperforin, extracts from *H. gentianoides*, and non-polar fractions of these extracts that are highly enriched in putative acylphloroglucinols, have anti-inflammatory bioactivity in mouse macrophage cells (Hillwig et al. 2008). *H. gentianoides* accumulates nine unusual specialized phytochemicals that have similar UV spectra, and are postulated to be acylphloroglucinols (Hillwig et al. 2008). Three of these compounds have been identified as saroaspidin A, uliginosin A, and hyperbrasilol C by NMR (Hillwig 2008).

Identifying the range of existing acylphloroglucinols and the routes to their synthesis will enhance our understanding of the development of diversity in biology, and inform the evolutionary origin of this branch of the polyketide pathway. In part because of their high-energy content and diverse chemical functionalities, polyketides in general provide a major potential as biorenewable chemicals to replace petroleum as a feedstock for polymer production (Nikolau et al. 2008, Oliver et al. 2009). Acylphloroglucinol research has particular potential for practical application in medicinal venues. For example, given the complex effects of hyperforin and other acylphloroglucinols on cellular and viral function,

the availability of a spectrum of acylphloroglucinols would better enable researchers to define structure-function relationships in humans and other animals.

Here, we determine the structures of the six previously unidentified major accumulating constituents in *H. gentianoides* as a monomeric and five dimeric acylphloroglucinols. We identify nine additional monomeric acylphloroglucinols that are implicated as precursors. Finally, we compare the accumulation patterns of these acylphloroglucinols between vegetative and reproductive phases of growth, and across two standardized accessions.

Materials and methods

Plant material

H. gentianoides germplasm (as seeds), accessioned in the US National Germplasm System as PI 664838 (and for studies in Figure 3a, Ames 27657), was obtained from the USDA North Central Regional Plant Introduction Station, Ames, Iowa. Accession PI 664838 of *H. gentianoides* provides the advantage that both vegetative and reproductive developmental phases are present on a given plant simultaneously under the growth conditions used in this study. For determinations of relative concentrations of acylphloroglucinols across shoot (leaf and stem, with flower buds for reproductive stage) material, plants were grown in randomized block design. Seeds were planted in a growing medium (Sunshine LC1 SUN-COIR, Sun Gro Horticulture, Canada) in a greenhouse with natural light supplemented by an 8/16 hr photoperiod at $120 \mu\text{mol m}^{-2} \text{s}^{-1}$, in temperatures between 19–25 degrees Celsius. Plants were watered daily, and fertilized bi-weekly. During each harvest, samples were collected beginning at 11 am. Vegetative shoots and reproductive shoots that had differentiated but had not begun to flower were collected from individual plants in triplicate (approximately 5–8 shoots per biological replicate). In experiments to evaluate seasonal variation, *H. gentianoides* seeds were planted in the greenhouse under the conditions described at four-month intervals, and grown 6 months, and harvested. (Plantings in September and January; harvests in February and June)

Plant samples were placed in a 1.5 mL Eppendorf tubes, and immediately stored in liquid nitrogen to minimize degradation of polyketides. Samples were kept in liquid nitrogen until they were extracted.

Preparation of extracts

Before extraction of each sample, liquid nitrogen was allowed to evaporate; then, the plant material was weighed without thawing and placed in a liquid nitrogen cooled mortar. 10 μL of apigenin in methanol at a concentration of 1 mg mL^{-1} was added to each sample as an internal standard. More liquid nitrogen was added to prevent thawing, and the mixture was finely ground with a pestle. 2 mL of methanol was added, the mixture was further ground, and then vortexed for 30 s, and centrifuged at 13 000 rpm for 2 min. The supernatant was decanted, filtered through a 0.45 micron nylon syringe filter, and the filtrate was placed under nitrogen gas to minimize oxidation of compounds that might be present at low concentrations and detected by mass spectrometry but not by a UV detector. Extracts were stored in a -80°C freezer until analysis.

High pressure liquid chromatography (HPLC)

HPLC conditions resembled those of Hillwig et al (2008). A Beckman Coulter Gold HPLC System (Fullerton, CA) with a Detector 168 photo diode array detector was used for determination of metabolite levels. A Synergi Max-RP 4 micron $150 \times 4.6 \text{ mm}$ column (Phenomenex, Torrance, CA 90501) was used for separation. A 10 mM ammonium acetate (solvent A) and 9:1 v/v acetonitrile/methanol (solvent B) mobile phase was used. The

gradient consisted of 87% A/13% B in 10 min to 83% A/17% B, then to 100% B in 25 min and held for 5 min. The flow rate was 1.0 mL min⁻¹, the solvent gradient and flow rate was initially described by (Ganzera et al. 2002). All solvents and the apigenin internal standard used were HPLC grade (Sigma, St. Louis, MO).

Liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS)

Samples were placed under nitrogen gas to minimize oxidation of low-accumulating compounds that might be detected by mass spectrometry but not by a UV detector. An Agilent Technologies Ion Trap 1100 was used for LC/ESI-MS analysis. Analytical separation was accomplished using a Synergi Max-RP 4 micron 150 × 4.6 mm column (Phenomenex, Torrance, CA 90501) with a 10 mM ammonium acetate (solvent A) and 9:1 v/v acetonitrile/methanol (solvent B) mobile phase, with a gradient from 85% A/15% B to 80% A/20% B over a 10 min time, to 100% B over 25 min, and held at 100% B for 5 minutes. The flow rate was 0.75 mL min⁻¹. All solvents were HPLC grade (Sigma, St. Louis, MO). This LC/ESI-MS analysis detected the nine final acylphloroglucinols but only two of eight precursors.

Metabolite quantification based on an internal standard

The relative concentrations of the acylphloroglucinols were quantified using HPLC-photo diode array UV, against 0.05 g (185.020 µmol) of apigenin (MW 270.24) as internal standard. Apigenin was added at the time of extraction to each biological replicate. Extracts derived from three independent biological replicates for each sample were analyzed, data normalized, converted to a relative scale of nmol based on the apigenin standard, and further normalized by dividing µmol of compound by the fresh weight of the plant material (µmol g⁻¹ FW).

Q-TOF mass spectrometry and spectral interpretation

High resolution mass spectrometric analysis was performed on a hybrid quadrupole orthogonal time-of-flight mass spectrometer (Waters SYNAPT G2 HDMS, Manchester, U.K.) equipped with an automatic chip-based nanoelectrospray source NanoMate HD (Advion BioSciences, Ithaca, NY). Ionization voltage was set to 1.09 kV, gas pressure to 1.45 psi in the negative-ion mode, and the source was controlled by C_{HIPOFT} 8.3.1 software (Advion BioSciences). All samples were diluted with 5 mM ammonium acetate in CHCl₃:MeOH:IPA (1:2:4, v/v/v) and infused. The system was equipped with an integral Advion LockSpray unit with its own reference sprayer that was controlled automatically by the acquisition software to collect a reference scan every 10 s, for 1 s. Reference internal calibrant (leucine enkephalin) was introduced into the lock mass sprayer at a constant flow rate of 3 µL min⁻¹ using an internal pump connected to the LockSpray. A single lock mass calibration at *m/z* 554.2615 in the negative-ion mode was used for the complete analysis. Spectral interpretation was performed with M_{ASS}L_{YNX}TM v.4.1 Mass Spectrometry Software (Waters Co., Milford, MA, USA). After peak picking, elemental formulas were calculated and assigned on the basis of *m/z* values within a 6 ppm error range. Standard conditions for plant metabolomics data (C_c H_h O_o, c 500, h 1000, o 20; -1.5 Double Bond Equivalent 50.0) were used in these calculations.

Results

Liquid chromatography/electrospray ionization-mass spectroscopy (LC/ESI-MS)-based structural identification of nine acylphloroglucinols of *H. gentianoides*

H. gentianoides (accessions Ames 27657 and Ames 28015) has been shown to contain multiple metabolites with retention times, mass spectra, and distinctive three-peaked UV

spectra indicative of acylphloroglucinols (Hillwig et al. 2008). Three of these compounds (MWs 446 D, 500 D, 554 D; ret times 23.418, 24.820, 24.993 min) have been previously identified in our lab as saraospidin A, uliginosin A, and hyperbrasilol C (Hillwig, 2008). These spectra were also compared to the 2-D NMR of the same compounds isolated from other species of *Hypericum*: saraospidin A, in *H. japonicum* (Ishiguro et al. 1987); uliginosin A, in *H. uliginosum* (Taylor and Brooker 1969); and hyperbrasilol C, in *H. brasiliense* (Rocha et al. 1995).

To further identify the chemical nature of the acylphloroglucinol-like compounds in *H. gentianoides*, plants of the accession PI 664838 were germinated from seeds, and grown in a greenhouse under natural plus supplemental light. Shoots in the vegetative phase of development were harvested from 11 months old plants (Figure 1). To minimize potential alteration or degradation of the polyketide constituents, we reduced the exposure of plant material and extracts to oxygen and *Hypericum* enzymes during pulverization, extraction and separation (Hillwig et al. 2008). The methanolic extract was analyzed by HPLC-UV and LC/ESI-MS (Figure S1, S2).

HPLC-UV of the *H. gentianoides* methanolic extract shows nine non-polar compounds with the characteristic three-peaked UV spectra with maxima at 220, 300, and 350 nm, indicative of acylphloroglucinols (Figure S1). Three of these nine compounds are saraospidin A, uliginosin A, and hyperbrasilol C, based on molecular mass, retention time, UV spectra, molecular fragmentation products, and NMR (Hillwig 2008). The extract was analyzed by LC/ESI-MS to further determine the structures of the nine acylphloroglucinols observed at m/z 's of 359, 497, 445, 459, 513, 499, 553, 567. Table 1 shows fragment ions and retention times from each MS/MS spectrum, of which are shown in Figure S1. Throughout this paper, there are several compounds that are newly identified, and do not have commonly used names; rather than make up a series of new names, these compounds will be referred to by abbreviations according to their chemical properties (Table 1 and Table 2).

MS/MS spectra, in negative ion mode, of *H. gentianoides* extract constituents saraospidin A, uliginosin A, and hyperbrasilol C indicate that the methylene bridge that connects the two decorated PIB molecules is the main fragmentation target (Table 1). The six additional acylphloroglucinols in *H. gentianoides* detected by LC/ESI-MS, which had not been previously characterized, were identified based on the following criteria: the MS/MS spectra for a diacylphloroglucinol should reveal the m/z of the two decorated PIB molecules composing said diacylphloroglucinol. For example, the MS/MS spectra in negative ion mode of hyperbrasilol C is composed of ions at 263, 277, and 289 m/z , corresponding to 3'-prenyl-PIB (3'prenPIB), 1'-prenyl-3'-methyl-4'-oxo-PIB (1'pren3'me4'oxoPIB), and 1'pren3'me4'oxoPIB with a methyl group fragment of the methylene bridge that connected the two monomers together, respectively. The fragmentation pattern of the three NMR identified compounds were used to establish an additional four compounds identity as: uliginosin B, 3,5-dihydroxy-4-isobutyryl-2-methyl-4-(3-methylbut-2-enyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-methylbenzyl)cyclohexa-2,5-dienone, (abbreviated as [3'mePIB]-[1'pren3'me4'oxoPIB]); 3,5-dihydroxy-4-4-dimethyl-2-(2-methylbutanoyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone (abbreviated as [3'3'4me6'oxoPIB]-[3'prenPIB]); and 3,5-dihydroxy-2-methyl-4-(3-methylbut-2-enyl)-4-(2-methylbutanoyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone (abbreviated as [1'pren3'4me4'oxoPIB]-[3'prenPIB]) (Table 1). The compound at RT=23.761 min is a diacylphloroglucinol which could be either saraospidin B or japonicin A; both compounds have the same molecular weight and MS/MS spectral pattern, and thus cannot be formally distinguished by MS/MS. NMR analysis was not performed on this compound, or the other five previously unidentified acylphloroglucinols in *H. gentianoides*, because of the difficulty in obtaining purified

compound necessary for crystallization. Saroaspidin B is a dimer of a phloroglucinol moiety (3',4-dimethyl-PIB (3'4mePIB), $m/z=223$) and a filicinic acid moiety (3',3'-dimethyl-6'-oxo-PIB (3'3'me6'oxoPIB), $m/z=223$), while japonicin A is a dimer of two filicinic acid moieties (3'3'me6'oxoPIB). Given that the other seven diacylphloroglucinols in *H. gentianoides* are each heterodimers composed of one phloroglucinol moiety (3'-methyl-PIB (3'mePIB), 3'4mePIB, 8-isobutyryl-2,2-dimethyl-chromene-5,7-diol (8ib22meC57diol), 3'prenPIB) and one filicinic acid moiety (3'3'me6'oxoPIB, 3',3',4-trimethyl-6'-oxo-PIB (3'3'4me6'oxoPIB), 1'pren3'me4'oxoPIB, 1'-prenyl-4,5'-dimethyl-4-oxo-PIB (1'pren3'4me4'oxoPIB), we view saroaspidin B as the more likely identity (Figure 2a). The first-eluting compound of the nine major acylphloroglucinols in *H. gentianoides* has the typical characteristic acylphloroglucinol UV absorbance spectrum, eluted at 21.566 minutes, and has a molecular weight of 360 D, $[M-H]^-$ ion at m/z 359. This compound, unlike the other eight predominant acylphloroglucinols, should be a *mono*acylphloroglucinol, because the minimum weight possible for a diacylphloroglucinol is 404 D. A modification to 1'pren3'4me4'oxoPIB, specifically a 3'-prenylation as observed in 3'prenPIB, could account for a molecular weight of 360. We predicted this compound to be 1',3'-diprenyl-4,5'-dimethyl-4'-oxo-PIB (1'3'pren45'me4'oxoPIB). Consistent with this interpretation, the MS/MS spectrum of this compound shows an intense fragmented ion at 222 m/z , an m/z of 222 corresponds to the core cyclic ring of this compound with 4 and 5' methyl groups but without 1' and 3' prenyl groups. We also expected a peak at $m/z=69$ corresponding to the fragmented prenyl group. However, possibly because of the low abundance of this ion (Table 1, Figure S2), we were unable to detect this peak through Q-TOF or LC.

One possible synthetic route for diacylphloroglucinols is that PIB is first decorated, followed by dimerization of the resultant monomer pool to form the diacylphloroglucinols. In this case, eight acylphloroglucinol monomers (synthesized from PIB) would be sufficient to account for all of the *H. gentianoides* diacylphloroglucinols, as a variety of combinations can be made (exemplified in Figure 2a). Furthermore, if the “monomer pool” biosynthetic route were correct, we might expect that there would be low levels of the eight acylphloroglucinol monomer precursors in *H. gentianoides* (Figure 2b).

An alternate process that could account for biosynthesis of the observed diacylphloroglucinols is that a single diacylphloroglucinol might be formed and decorated to form the other diacylphloroglucinols. If this “diacylphloroglucinol decoration” route were correct, we would anticipate the presence of the precursors: partially decorated diacylphloroglucinols, and the fully undecorated dimer (di-PIB) (Figure 2c).

Acylphloroglucinol Accumulation Differs Between Life Phases and Accessions of *H. gentianoides*—

To facilitate detection of acylphloroglucinol precursors, which might be present at lower abundance or be more difficult to detect by the methods used, we wanted *H. gentianoides* material with high acylphloroglucinol concentrations and that was relatively easy to obtain. To evaluate which plant material might have higher levels of acylphloroglucinols, the concentrations of major acylphloroglucinols were compared under different conditions of plant growth. For these analyses, we used accessions of *H. gentianoides* (Ames 27657 and PI 664838) whose seeds are being maintained within the US National Plant Germplasm System.

First, we determined whether acylphloroglucinol accumulation in *H. gentianoides* varied with the season of the year, using the greenhouse growth conditions we had previously established for optimal plant growth. Under these conditions, supplemental light was used to create similar 12-hour daily photoperiods throughout the year. To determine whether the season affected the level of accumulation of acylphloroglucinols, batches of *H. gentianoides*

were planted at four-month intervals, allowed to grow six months, and vegetative shoots were harvested (harvests in February and June). The growth and morphology of these greenhouse-grown *H. gentianoides* plants was not visibly distinguishable, irrespective of the growth season. A comparison of acylphloroglucinol accumulation in February versus June is shown in Figure S3. The slope of the regression between the two time points was 1.01 and an R-squared value of 0.994. Thus, under these growth conditions, both the development of the *H. gentianoides* and the relative concentrations of each of its predominant acylphloroglucinols are similar across these dates.

Relative concentrations of acylphloroglucinols in *H. gentianoides* accession PI 664838 was compared at three stages of development (seedling, mature vegetative, and flowering reproductive) (Figure 1, 3a; Table S1). Reproductive phase shoots (stem, leaves and flower buds) and vegetative phase shoots (stem and leaves) were harvested from plants that had both of these stages simultaneously. Thus, levels of acylphloroglucinol accumulation were determined for vegetative and reproductive phase shoots from the same plants. The vegetative phase shoots accumulate higher amounts of all diacylphloroglucinols ($p < 1.9 \times 10^{-6}$ to $p < 0.03$) in μmol per gram fresh weight tissue ($\mu\text{mol g}^{-1}$ FW) (Figure 3b); the least abundant major accumulating constituent, 1'3'pren45'me4'oxoPIB, is more than 10-fold higher in the vegetative phase ($p < 0.055$).

Seedling shoots from the two standardized accessions Ames 27657 and PI 664838 were evaluated for acylphloroglucinols (Figure 3b, Table S1). PI 664838 accumulated between 2 to 7.5 fold higher amounts of each of the nine major acylphloroglucinols ($p < 0.0005$ to $p < 0.039$) than did Ames 27657. The greater acylphloroglucinol accumulation in seedling shoots and in the mature vegetative shoots of PI 664838, together with the shorter time to harvest of seedlings, led us to use seedling shoots from accession PI 664838 for further identification of *H. gentianoides* acylphloroglucinols.

Hybrid quadrupole orthogonal time-of-flight mass spectrometry (Q-TOF) detects PIB and eight other monoacylphloroglucinols

Determination of the complement of mono- and diacylphloroglucinols present in *H. gentianoides* could provide evidence for the biosynthetic route to the major accumulating acylphloroglucinols. Because LC/ESI-MS did not reveal potential diacylphloroglucinol intermediates, we chose a more sensitive method, hybrid quadrupole orthogonal time-of-flight mass (Q-TOF) spectrometry. This method not only detects molecules that might be undetectable using LC/ESI-MS, but also yields precise data on mass (up to 10^{-4} of a dalton).

To experimentally determine whether the eight acylphloroglucinol monomers that we postulated to be precursors of the diacylphloroglucinols in *H. gentianoides* are actually present in that species, high diacylphloroglucinol-accumulating seedlings of PI 664838 were extracted in methanol, and these extracts were diluted with 5 mM ammonium acetate in $\text{CHCl}_3\text{:MeOH:IPA}$ and analyzed by Q-TOF. All nine of the acylphloroglucinols detected by LC/ESI-MS (Table 1) were also detected by Q-TOF spectrometry (Table 2).

Q-TOF spectrometry also showed compounds that could not be detected by LC/ESI-MS. Notably, molecular weights and empirical formulas corresponding to all eight of the monoacylphloroglucinols that could explain synthesis of the *H. gentianoides* major accumulating acylphloroglucinols were identified, as was the precursor PIB and two putative partially decorated dimers (Figure 2). To provide additional evidence as to the identity of the putative monomers and partially decorated dimers, we employed Q-TOF-MS/MS on these masses (Figure S4). Because of the low abundance of MS/MS fragmentation ions at m/z values corresponding to the predicted monomers 3'4mePIB and 8ib22meC57diol, these two compounds were not included in this analysis. Using MS/MS

itself does not positively identify these compounds, however, we have been able to leverage NMR data from saroaspidin A and hyperbrasilol C, which are composed of monomers 3'mePIB, 3'3'me6'oxoPIB, 3'prenPIB, and 1'pren3'me4'oxoPIB collectively (Ishiguro et al. 1987, Rocha et al. 1996; Hillwig 2008), for the identification. Our MS/MS analyses indicate the methylene bridge is the primary fragmentation target, yielding 3'mePIB and 3'3'me6'oxoPIB from saroaspidin A as well as 3'prenPIB and 1'pren3'me4'oxoPIB from hyperbrasilol C (Figure S2C, 2H). These ions were each fragmented again, and Q-TOF-MS/MS was employed to identify the fragmentation patterns of 3'mePIB, 3'3'me6'oxoPIB, 3'prenPIB, and 1'pren3'me4'oxoPIB. These, in turn, were directly compared to the Q-TOF-MS/MS profiles corresponding to the predicted 3'mePIB, 3'3'me6'oxoPIB, 3'prenPIB, and 1'pren3'me4'oxoPIB. Identical fragmentation patterns confirm that the Q-TOF-MS derived masses are indeed 3'mePIB, 3'3'me6'oxoPIB, 3'prenPIB, and 1'pren3'me4'oxoPIB (Figure 4, Table 3).

MS/MS analysis of these monomers indicates an isobutane is fragmented from the isobutyl group in 3'mePIB, 3'3'me6'oxoPIB, 3'prenPIB, and 1'pren3'me4'oxoPIB. This is the only fragmentation, with the exception of the molecular ion, present in MS/MS spectra of 3'mePIB, and 3'3'me6'oxoPIB. MS/MS spectra for 3'prenPIB have four ions present at $m/z=151$, 194, 219, and 263 (the molecular ion). The ion at 151 m/z corresponds to fragmentation of the isobutane and prenyl group, while 194 m/z corresponds to fragmentation of only the prenyl group, and 219 m/z is a fragmentation of only the isobutane. MS/MS spectra of 1'pren3'me4'oxoPIB follow the same pattern of fragmentation as 3'prenPIB, in which the isobutyl and prenyl groups are the primary target, yielding ions at 166, 208, and 233 m/z (Figure S4). We cannot identify 3'4mePIB, 3'3'4me6'oxoPIB, 8ib22meC57diol, or 1'pren3'4me4'oxoPIB with as great a certainty as 3'mePIB, 3'3'me6'oxoPIB, 3'prenPIB, and 1'pren3'me4'oxoPIB, but the ion at $m/z=291$ is likely 1'pren3'4me4'oxoPIB, given the primary fragmentation ion of this compound is at $m/z=222$, exactly the same mass as seen in MS/MS spectra of 1'3'pren45'me4'oxoPIB. This is expected given $m/z=222$ would correspond to a fragmentation of one prenyl group from 1'pren3'4me4'oxoPIB and two fragmented prenyl groups from 1'3'pren45'me4'oxoPIB. Suggestive MS/MS spectra for 1'pren3'4me4'oxoPIB along with the confirmed presence of 3'mePIB, 3'3'me6'oxoPIB, 3'prenPIB, and 1'pren3'me4'oxoPIB indicate the m/z do indeed correspond to the monomers. The monomers that we observed were probably not detected in previous studies of *Hypericum* spp that contain diacylphloroglucinols (Taylor et al. 1969, Ishiguro et al. 1987, Rocha et al. 1996, Huang et al. 2011) because of the detection method or their lower abundance. Also identified by Q-TOF-MS/MS were the partially decorated dimers [3'3'me6'oxoPIB]-[PIB] and [1'pren3'me4'oxoPIB]-[PIB] (Table 3, Figure 2C). These partially decorated dimers are fragmented at the methylene bridge in the same way as observed for the fully decorated dimers; yielding fragmentation ions at 195 and 223 m/z for [3'3'me6'oxoPIB]-[PIB] and 195 and 277 m/z for [1'pren3'me4'oxoPIB]-[PIB] (Figure S4).

Discussion

The mono- and diacylphloroglucinols thus far identified from *Hypericum* spp. display a wealth of structural variety (Avato et al. 2004, Gibbons et al. 2005, Manning et al. 2011). A number of these compounds have been shown to induce diverse bioactivities when applied to other organisms (Schempp et al. 2002, Avato et al. 2004, Mennini et al. 2004, Beerhues 2006, Hillwig et al. 2008, Henry et al. 2009, Saddiqe et al. 2010, Wang et al. 2011, Stein et al. 2012, Zaher et al. 2012). St. John's Wort contains a single monoacylphloroglucinol that has been the subjects of hundreds of medicinal studies (hyperforin), but no known diacylphloroglucinols. The biological activities of diacylphloroglucinols are a recent research focus. Uliginosin B has anti-depressive effects when given to mice before a forced swim stress test (Stein et al. 2012). Saroaspidin A, B, uliginosin A, B, and hyperbrasilol C

have anti-bacterial activity properties in *Staphylococcus aureus*, and *Bacillus cereus* assays (Ishiguro et al. 1987, Rocha et al. 1995, Rocha et al. 1996, Winkelmann 2001, França and Kuster 2009). Diacylphloroglucinols can show specificity with respect to a given activity; for example, saroaspidin A is active in anti-inflammatory assays with mouse macrophage cells, while uliginosin A has little anti-inflammatory activity in these assays (Hillwig et al. 2008). These multiple bioactive effects, combined with the specificity of the action of individual acylphloroglucinols, emphasize the scope of potential medical applications for these compounds.

The major acylphloroglucinols of *H. gentianoides* are a single monoacylphloroglucinol and eight diacylphloroglucinols. Three of the diacylphloroglucinols, [3'mePIB]-[1'pren3'me4'oxoPIB], [3'3'4me6'oxoPIB]-[3'prenPIB], [1'pren3'4me4'oxoPIB]-[3'prenPIB], had not been previously described in any species. Identifying the acylphloroglucinol constituents of *H. gentianoides* is a prerequisite to deciphering the pathways by which they are biosynthesized. Ultimately, such data will be critical for identifying the primary sequences and three-dimensional structures of enzymes and regulatory proteins that control this pathway, and will provide an important step towards understanding that pathway in the context of its evolution (Schmidt et al. 2003, O'Maille et al. 2008, Ngaki et al. 2012).

From an applied aspect, the determination of the chemical nature of *Hypericum* polyketide constituents is important for those who use extracts of this genus as medical supplements. Furthermore, the availability of a wide variety of acylphloroglucinols will provides researchers with tools to investigate the mechanisms by which members of this class of compounds affect other organisms, and to dissect the enzymes and regulatory factors that lead to these compounds in plants. This information sets the stage for genetic engineering of custom acylphloroglucinols (Crispin and Wurtele 2013; <http://www.metnetdb.org/pmr/>) and other polyketides, which is significant because of the potential medicinal applications.

Virtually nothing had been reported previously about the pathway leading to the biosynthesis of diacylphloroglucinols. Here, we combine previous knowledge and our new understanding of the acylphloroglucinols in *H. gentianoides* to propose a pathway leading to the biosynthesis of diacylphloroglucinols (Figure 5) and describe a potential mechanism for dimerization (Figure 6).

Biosynthesis of the monoacylphloroglucinol hyperforin in *H. perforatum* is becoming better understood (Beerhues 2003, Soelberg et al. 2007, Karppinen 2010) and provides a possible template for the initial steps of diacylphloroglucinol biosynthesis (Figure 5). PIB could be derived from either pyruvate or valine in *H. perforatum* (Adam et al. 2002). Isotopically labeled L-[U-¹³C₆]-valine was preferentially incorporated into the acyl chain of hyperforin in liquid cultures of 12 day old *H. perforatum*, indicating that valine is likely the major precursor under this condition (Karppinen 2010). In *H. perforatum*, PIB is an intermediate of hyperforin (Paniego et al. 1999, Adam et al. 2002, Soelberg et al. 2007, Karppinen 2010) via its prenylation to form 3'prenPIB (Adam et al. 2002, Karppinen et al. 2007). Presumably the early pathway to acylphloroglucinols is common among *Hypericum* spp, consistent with this supposition, we identified PIB and 3'prenPIB in *H. gentianoides*. Either of two processes could yield the array of diacylphloroglucinols present in *H. gentianoides*. 1) PIB monomers might be decorated, and the pool of decorated monomers then dimerized (decoration of monomer pool); and/or 2) PIB monomers might be dimerized and then the dimers might be further modified (decoration of dimers)

Our observations, taken together, indicate that the synthesis of diacylphloroglucinols likely occurs via a pool of decorated monomers (Figure 5). PIB and eight other

monoacylphloroglucinols, each a derivative of PIB, were detected in *H. gentianoides*. The *H. gentianoides* monomers, in different combinations, would exactly explain the presence of each of the diacylphloroglucinols. Thus, the particular complement of acylphloroglucinol monomers supports the “monomer pool” concept, in which the monomers are first decorated and then dimerized. We could not detect any completely undecorated dimers, nor six of the eight partially decorated dimers that would be expected if diacylphloroglucinol biosynthesis occurred by a process in which an “undecorated” acylphloroglucinol dimer is biosynthesized, followed by a stepwise derivatization. (We did, however, detect low concentrations of two of the eight partially decorated dimers, that would be anticipated if the “decoration of dimers” process took place, [3'3'me6'oxoPIB]-[PIB] and [1'3'pren3'me4'oxoPIB]-[PIB].) The presence of substantial concentrations of 1'3'pren4'5'me4'oxoPIB lends further support for the “monomer pool” concept. This acylphloroglucinol is detectable through every method we have tested, and is not able to be dimerized as the available carbons are derivatized. It would be rather convoluted to consider that, as would be predicted by the decoration of dimer model, the plant would first dimerize two PIB molecules, decorate them, break them apart, and then prenylate the fragmented half as would be necessary to synthesize this monoacylphloroglucinol. The idea that one monomer becomes fully decorated and thus accumulates as such, as would be predicted by the decoration of monomer pool model, is much more direct.

A notable difference between hyperforin and diacylphloroglucinol biosynthesis is the creation of a methylene bridge in diacylphloroglucinols. There is no reported mechanism describing this reaction, however, the mechanism for EC 1.21.3.3, which forms a methylene bridge during the formation of (S)-scoulerine from (S)-reticuline (Kutchan and Ditttrich 1995), has been described. EC 1.21.3.3 has features in common with the dimerization of acylphloroglucinols, as both involve formation of a methylene bridge between two phenolic rings. Applying the EC 1.21.3.3 mechanism to diacylphloroglucinol formation, the enzymatic synthesis of a methylene bridge between two PIB molecules could proceed as shown in Figure 6.

In summary, we characterize the mono- and diacylphloroglucinols of *H. gentianoides*, and describe their accumulation in different biological accessions and developmental stages. The data is deposited in the public PMR metabolomics database (<http://metnetdb.org/PMR/>, Hur et al. 2013), enabling accessibility and extension by the research community. The data lead us to propose a network for acylphloroglucinol biosynthesis in which PIB is decorated to yield the observed monomers, followed by condensed in various combinations, giving rise to the observed major constituents. These data enhance our understanding of the diversity of this medicinal species, and set the stage for characterization of the genes and enzymes involved in acylphloroglucinol metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PIB	phlorisobutyrophenone
1'pren3'me4'oxoPIB	1'-prenyl-3'-methyl-4'-oxo-phlorisobutyrophenone
1'pren3'4me4'oxoPIB	1'-prenyl-3',4-dimethyl-4'-oxo-phlorisobutyrophenone
[1'pren3'4me4'oxoPIB]-[3'prenPIB]	3,5-dihydroxy-2-methyl-4-(3-methylbut-2-enyl)-4-(2-methylbutanoyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone
1'3'pren45'me4'oxoPIB	1',3'-diprenyl-4,5'-dimethyl-4'-oxo-phlorisobutyrophenone
3'mePIB	3'-methyl-phlorisobutyrophenone
[3'mePIB]-[1'pren3'me4'oxoPIB]	3,5-dihydroxy-4-isobutyryl-2-methyl-4-(3-methylbut-2-enyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-methylbenzyl)cyclohexa-2,5-dienone
3'4mePIB	3',4-dimethyl-phlorisobutyrophenone
3'3'me6'oxoPIB	3',3'-dimethyl-6'-oxo-phlorisobutyrophenone
3'3'4me6'oxoPIB	3',3',4-trimethyl-6'-oxo-phlorisobutyrophenone
[3'3'4me6'oxoPIB]-[3'prenPIB]	3,5-dihydroxy-4,4-dimethyl-2-(2-methylbutanoyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone
3'prenPIB	3'-prenyl-phlorisobutyrophenone
LC/ESI-MS	liquid chromatography/electrospray ionization-mass spectrometry
MW	molecular weight
RT	retention time
Q-TOF	quadrupole orthogonal time-of-flight
FW	fresh weight

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Figure 1. Developmental phases of *H. gentianoides* life cycle. In some accessions of *H. gentianoides*, including PI 664838, both vegetative and reproductive phases often occur concurrently on a single plant.

Figure 2.

Diacylphloroglucinols in *Hypericum gentianoides*. A) Each diacylphloroglucinol identified in *H. gentianoides* (see Table 1) is composed of one phloroglucinol (purple) and one filicinic acid (green); many of these diacylphloroglucinols share a common phloroglucinol or filicinic acid. For example, Saroaspidin A, Saroaspidin B, Uliginosin A, and Uliginosin B, all share the filicinic acid 3',3'-dimethyl-6'-oxo-phlorisobutyrophenone (3'3'me6'oxoPIB). B) Four PIB derivatives from *H. gentianoides* identified by Q-TOF. Methylation and/or prenylation of PIB would give rise to each of these compounds. Additional methylation and/or cyclization of the resultant compounds would give rise to four more monomers, creating a pool of eight monomers sufficient to explain the synthesis of all nine highly accumulated acylphloroglucinols, without any post-dimerization modifications. C) Nine diacylphloroglucinols would be possible if diacylphloroglucinol biosynthesis occurred via a "diacylphloroglucinol decoration" route. In black are compounds that were identified through our Q-TOF analyses, in red are compounds that were not detected in our Q-TOF analysis of *H. gentianoides* extracts. The fully undecorated dimer (diPIB), and six of the eight partially decorated dimers that would be required if synthesis followed the "diacylphloroglucinol decoration" route were not detected. We assume the two partially decorated dimers present in *H. gentianoides* are 1) degradation products of diacylphloroglucinol end products or 2) products resulting from dimerization of a decorated monomer to a PIB molecule.

Figure 3.

Accumulation of acylphloroglucinols in *Hypericum gentianoides* varies with developmental stage and accession. HPLC analysis was used for relative quantification of the nine major acylphloroglucinol metabolites. Three biological replicates were used for each sample. Data is analyzed and visualized in the interactive Plant Metabolomics Resource (www.metnetdb.org/pmr). Complete data is shown in Table S1. Volcano plots illustrates a comparison of two of the most diverse materials. In this plot, the Y-axis represents the p-value (lower p-values indicate a more significant difference); the X-axis represents the fold change (the farther from 1, the greater the fold-change). p-values are determined using a student T-test after log (base 2) transformation. 1-1'3'pren45'me4'oxoPIB; 2-Uliginosin B; 3-Saroaspidin A; 4-Saroaspidin B; 5-[3'mePIB]-[1'pren3'me4'oxoPIB]; 6-[3'3'4me6'oxoPIB]-[3'prenPIB]; 7-Uliginosin A; 8-Hyperbrasilol C; 9-[1'3'pren45'me4'oxoPIB]-[3'prenPIB]. (a) Accumulation of 9 major acylphloroglucinols is between 2–7.5 fold greater in young seedlings of standardized accession PI 664838 compared to Ames 27657 young seedlings. (n=3, p values range from 0.0009 to 0.05). (b) Accumulation of 9 major acylphloroglucinols (is between 1.7–10.9 fold greater in vegetative phase shoots than in reproductive phase shoots (preflowering) in the standardized accession PI 664838). (n=3, p values range from 1.9×10^{-6} to 0.03)

Figure 4.

Q-TOF-MS/MS/MS of *H. gentianoides* extract to validate the identity of the predicted monomers of Table 2. The fragmentation pattern corresponding to 3'mePIB, 3'3'me6'oxoPIB, 3'prenPIB, and 1'pren3'me4'oxoPIB was compared to that of the previously identified uliginosin A, saroaspidin A, and hyperbrasilol C. Identical fragmentation patterns indicate that these masses in fact correspond to these four monomers and not any other isomer. MS/MS spectra at the molecular weights of the putative monomers 3'3'4me6'oxoPIB and 1'pren3'4me4'oxoPIB were analyzed (Table 3). None of the previously identified acylphloroglucinols contain either of these two monomers. Therefore these fragmentation patterns are consistent with the presence of 3'3'4me6'oxoPIB and 1'pren3'4me4'oxoPIB in *H. gentianoides*.

Figure 5.

Proposed biosynthetic pathway of acylphloroglucinols in *Hypericum gentianoides* as detected by Q-TOF-MS, Q-TOF-MS/MS and LC/ESI-MS analysis. All acylphloroglucinols involved in the biosynthesis of the nine acylphloroglucinol products are shown in this schematic. Valine and possibly pyruvate are likely primary metabolite precursors of acylphloroglucinols in *H. perforatum* (Karppinen 2010). Q-TOF and LC/ESI analysis indicates that *H. gentianoides* contains PIB and PIB derivatives that are decorated by prenylation or methylation, or are cyclized (in the case of 8ib22meC57diol). Every acylphloroglucinol dimer in *H. gentianoides* can be accounted for as a dimerization product of one of the detected phloroglucinol monomers and one of the detected filicinic acid

monomers. Thus, the complement of monomers in *H. gentianoides* is consistent with the concept that monomers are first decorated and then dimerized to yield the diacylphloroglucinols. Blue, metabolites identified for the first time in this study; rust, identified in *H. gentianoides* (this study) and other *Hypericum* species (see Table 2). Hyperforin (grey) accumulates in *H. perforatum* (Soelberg et al. 2007). PIB* and 3'prenPIB* are postulated to be a hyperforin precursor in *H. perforatum* (Adam et al. 2002).

Figure 6.

Proposed mechanism of methylene bridge formation in diacylphloroglucinol biosynthesis. The formation of a diacylphloroglucinol from two monoacylphloroglucinols proceeds via a multi-step process similar to that of reticuline oxidase (Kutchan and Dittrich 1995). First one of the monomers would have to be methylated, that methyl group would become charged. A nucleophilic attack of this charged methyl group by the other monomer would form the observed methylene bridge. A third basic residue would assist in stabilizing the second monomer completing the observed diacylphloroglucinols. R_1 and R_2 are chemical groups corresponding to PIB decorations. B_1 , B_2 , and B_3 are proposed basic residues of the enzyme involved in diacylphloroglucinol formation.

Table 1

Molecular and MS/MS ions of acylphloroglucinols from *H. gentianoides* using LC/ESI-MS.

Blue, metabolites identified for the first time in this study. Rust, identified in *H. gentianoides* (this study)

and other *Hypericum* species

Relative Retention Time (min)	Abbreviation/common name	<i>m/z</i>	Fragment Ions
21.566	1'3'pren45'me4'oxoPIB	359	222
23.32	Uliginosin B	497	223, 235, 261
23.418	Saroaspidin A	445	209, 223, 235
23.791	Saroaspidin B	459	223, 235
24.090	[3'mePIB]-[1'pren3'me4'oxoPIB]	499	209, 221, 277, 289
24.510	[3'3'4me6'oxoPIB]-[3'prenPIB]	513	237, 249, 263, 275
24.820	Uliginosin A	499	223, 235, 263, 275
24.993	Hyperbrasilol C	553	263, 277, 289
25.265	[1'3'pren45'me4'oxoPIB]-[3'prenPIB]	567	263, 291, 303

^aLC/ESI-MS analysis. Molecular ions measured in negative-ion mode. Vegetative phase shoots from 11-month-old plants of *H. gentianoides* accession PI 664838. RT, retention time.

^bSaroaspidin B and japonicin A have the same MW and MS/MS spectra and cannot be formally distinguished by our analysis. Japonicin A is a homodimer of two filicinic acid moieties, while saroaspidin B is a heterodimer. Because the other seven diacylphloroglucinols are heterodimers of a phloroglucinol and filicinic acid moiety we believe saroaspidin B to be the more likely compound.

Table 2

Molecular ions, predicted empirical formulas, and designations of acylphloroglucinols and their putative precursors present in *H. gentianoides*, as identified by high-resolution Q-TOF mass analysis.

Blue, metabolites identified for the first time in this study. Rust, identified in

H. gentianoides (this study) and other *Hypericum* species

Relative Abundance ($\mu\text{mol g}^{-1}$ FW) *	Predicted m/z	Measured m/z	Predicted Empirical Formula	Mass Difference (ppm)	Systematic Name	Compound Abbreviation/Common Name
.0706	195.07	195.0657	C10 H11 O4	0.0	2',4',6'-trihydroxy-isobutyrophenone	PIB/Phlorisobutyrophenone <i>1,2</i> this study
.3844	209.08	209.0816	C11 H13 O4	1.0	3'-methyl-phlorisobutyrophenone	3' mePIB this study
.3516	223.10	223.0972	C12 H15 O4	0.9	3',3'-dimethyl-6'-oxo-phlorisobutyrophenone or 3' 4-dimethyl-phlorisobutyrophenone	3'3' me6' oxoPIB or 3'4 mePIB this study
.0756	237.11	237.1128	C13 H17 O4	0.4	3',3',4'-trimethyl-6'-oxo-phlorisobutyrophenone	3'3'4 me6' oxoPIB this study
.0693	261.11	261.1219	C15 H17 O4	---	8-isobutyryl-2,2-dimethyl-chromene-5,7-diol	8ib22meC57diol this study
.5344	263.13	263.1285	C15 H19 O4	0.8	3'-prenyl-phlorisobutyrophenone	3prenPIB <i>4</i> this study
.4525	277.14	277.1442	C16 H21 O4	0.7	1'-prenyl-3'-methyl-4'-oxo-phlorisobutyrophenone	1'pren3' me4' oxoPIB this study
.0429	291.16	291.0848	C17 H23 O4	---	1'-prenyl-3',4'-dimethyl-4'-oxo-phlorisobutyrophenone	1'pren3'4 me4' oxoPIB this study
.0403	359.22	359.0747	C22 H31 O4	---	1',3'-diprenyl-4,5'-dimethyl-4'-oxo-phlorisobutyrophenone	1'3' pren45' me4' oxoPIB this study
.7890	445.18	445.1862	C24 H29 O8	0.0	3,5-dihydroxy-2-isobutyryl-4,4-dimethyl-6-(2,4,6-trihydroxy-3-isobutyryl-5-methylbenzyl)cyclohexa-2,5-dienone	Saroaspidin A <i>2,5</i> , this study
.2344	459.20	459.2019	C25 H31 O8	0.0	3,5-dihydroxy-2-isobutyryl-4,4-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-(2-methylbutanoyl)benzyl)cyclohexa-2,5-dienone	Saroaspidin B <i>2</i> , this study
.0706	497.22	497.2226	C28 H33 O8	-1.6	2-((5,7-dihydroxy-8-isobutyryl-2,2-dimethyl-2H-chromen-6-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4,4-dimethylcyclohexa-2,5-dienone	Uliginosin B <i>1, 6</i> , this study

Relative Abundance ($\mu\text{mol g}^{-1}$ FW)*	Predicted <i>m/z</i>	Measured <i>m/z</i>	Predicted Empirical Formula	Mass Difference (ppm)	Systematic Name	Compound Abbreviation/Common Name
.0416	499.23	499.0497	C28 H35 O8	---	3,5-dihydroxy-4-isobutyl-2-methyl-4-(3-methylbut-2-enyl)-6-(2,4,6-trihydroxy-3- <i>l</i> -isobutyl)-5-methylbenzyl)cyclohexa-2,5-dienone	[3'-mePIB]- [1'-pren3'-4me4'-oxoPIB] this study
3.437	499.23	499.2330	C28 H35 O8	-0.4	3,5-dihydroxy-2-isobutyl-4,4-dimethyl-6-(2,4,6-trihydroxy-3-isobutyl)-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone	Uliginosin A <i>1</i> , <i>5'</i> this study
.5092	513.25	513.2330	C29 H37 O8	-1.0	3,5-dihydroxy-4,4-dimethyl-2-(2-methylbutanoyl)-6-(2,4,6-trihydroxy-3-isobutyl)-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone	[3'-3'-4me6'-oxoPIB]-[3'-prenPIB] this study
2.103	553.28	553.2800	C32 H41 O8	-0.2	3,5-dihydroxy-4-isobutyl-2-methyl-4-(3-methylbut-2-enyl)-6-(2,4,6-trihydroxy-3-isobutyl)-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone	Hyperbrasilol C <i>3'</i> this study
.0756	567.29	567.2965	C33 H43 O8	1.2	3,5-dihydroxy-2-methyl-4-(3-methylbut-2-enyl)-4-(2-methylbutanoyl)-6-(2,4,6-trihydroxy-3-isobutyl)-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone	[1'-3'-pren4'-5'-me4'-oxoPIB]- [3'-prenPIB] this study

* Relative abundance based on an apigenin internal standard.

1 Taylor and Brooker, 1969;

2 Ishiguro et al., 1987;

3 Rocha et al., 1996;

4 Adam et al., 2002;

5 Huang et al., 2011;

6 Stein et al., 2012.

Abbreviation: phloroisobutyrophenone=PIB; methyl=me; pren=prenyl; oxo=oxo; ib=isobutyl; C=chromene

Table 3

Molecular ions and MS/MS of putative monomers and partially decorated diacylphloroglucinols from *H. gentianoides* detected using Q-TOF. With the exception of 3'3'4me6'oxoPIB and 1'pren3'me4'oxoPIB, an isobutane is fragmented from the isobutyl group on all monomers. MS/MS of 3'prenPIB and 1'pren3'me4'oxoPIB show a fragmentation on the prenyl group as well. 3'4mePIB and 8ib22meC57diol are not shown due to low ion abundance. The partially decorated dimers (3'3'me6'oxoPIB and PIB and 1'pren3'me4'oxoPIB and PIB) are fragmented at the methylene bridge connecting them, similar to what is observed in the full decorated dimers.

Blue, metabolites identified for

the first time in this study. Rust, identified in *H. gentianoides* (this study) and other *Hypericum* species

Compound Abbreviation	<i>m/z</i>	Fragment Ions (<i>m/z</i>)
PIB	195	111, 151
3'mePIB	209	165, 209
3'3'me6'oxoPIB	223	179, 223
3'3'4me6'oxoPIB	237	193, 237
3'prenPIB	263	151, 194, 219, 263
1'pren3'me4'oxoPIB	277	166, 208, 233
1'pren3'4me4'oxoPIB	291	222
[3'3'me6'oxoPIB]-[PIB]	431	195, 223
[1'pren3'me4'oxoPIB]-[PIB]	485	195, 277