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Phenylpropanoid glycosides of *Mimulus guttatus* (yellow monkeyflower)

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ABSTRACT

Yellow monkeyflower [*Mimulus guttatus* DC., (Phyrmaceae)] has long been a model plant species for studies in genetics, evolution, and ecology, including plant–animal interactions. Nonetheless, exceedingly little is known about its secondary chemistry. We have discovered that the foliage of yellow monkeyflower contains a diverse suite of phenylpropanoid glycosides (PPGs); a class of compounds with many known biological activities. Using ^{1}H and ^{13}C NMR and UV and MS chromatography techniques, we positively identified five PPGs from the leaves of yellow monkeyflower. Four of these compounds occur in other species and one is previously undescribed. We also present UV and high-resolution tandem MS data that putatively identify 11 additional foliar compounds as PPGs. This initial discovery and elucidation of yellow monkeyflower's secondary chemistry will be important for continued study of the genetics and ecology of this model species.

1. Introduction

Yellow monkeyflower [*Mimulus guttatus* DC. (Phyrmaceae)] has emerged as a model system for integrated studies of genetics, evolution, and ecology, including plant-insect interactions (Eubanks et al., 2005; Fenster and Ritland, 1994; Hall and Willis, 2005; Holeski et al., 2013; Mojica et al., 2012; Wu et al., 2008). Yellow monkeyflower protects itself from herbivores with physical defenses in the form of trichomes, which have been well studied (Holeski, 2007; Holeski et al., 2010; Scoville et al., 2011). Similarly, the foliar surface secondary compounds of plants in this genus have been explored in several studies. For instance, Bohm (1992) described flavonoids from the leaf exudate of *Mimulus lewisii* and both Lincoln and Walla (1986) and Hare (2002a, 2002b) found that the surface leaf resins of *Mimulus aurantiacus* contain a variety of geranylflavanones and an α-pyrone. Additional work with ground leaf extracts identified compounds similar to these leaf resins in other *Mimulus* species (Piovano et al., 2009; Salem et al., 2011). In contrast, other than a report of the compound responsible for the yellow flower color (Nitsche et al., 1969), very little is known about the internal secondary metabolism of yellow monkeyflower (Holeski et al., 2013) or any other species in the genus. Absent a thorough understanding of the signature secondary metabolites of *M. guttatus*, its utility as a model species for studies of ecological and evolutionary interactions is limited.

We have discovered that yellow monkeyflower synthesizes a suite of mono- and disaccharide phenylpropanoid glycosides (PPGs; aka, phenylethanoid glycosides, Jimenez and Riguera, 1994; or caffeic acid esters, Mølgaard and Ravn, 1988) in its foliage. PPGs originate from the shikimic acid-phenylpropanoid pathway and include simple monosaccharides, consisting of hydroxycinnamic acid and hydroxyphenylethyl moieties bonded to a central β-glucopyranose by ester and glycosidic linkages, respectively, and more complex di- and trisaccharides with one or two additional sugars linked to the core glucose (Jimenez and Riguera, 1994; Mølgaard and Ravn, 1988). Members of this compound class have shown a wide range of biological activity, including inhibition of plant pathogenic bacteria and fungi (Ravn et al., 1989), antioxidant

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activity (Cardinali et al., 2012; Jimenez and Riguera, 1994; Owen et al., 2003), tumor cell suppression (Chen et al., 2002; Jimenez and Riguera, 1994), feeding stimulation of specialist herbivores (Holeski et al., 2013), and deterrence of generalist insects (Cooper et al., 1980; Mølgaard, 1986). In addition, recent work has shown that these compounds can vary considerably among natural populations and between annual and perennial ecotypes of yellow monkeyflower (Holeski et al., 2013).

We used various liquid chromatography techniques to isolate and purify these compounds, and then identified them with a combination of ^{1}H and ^{13}C NMR and high-resolution tandem mass spectrometry. The characterization of this group of secondary metabolites in yellow monkeyflower represents an important advance in the continued study of plant-insect interactions with this important model species.

2. Results and discussion

Almost all plant species with available genomes and prominent secondary metabolism have well-characterized chemistry. For instance, *Arabidopsis thaliana* contains glucosinolates (Shroff et al., 2008), black cottonwood (*Populus trichocarpa*) produces phenolic glycosides (Boeckler et al., 2011), and Norway spruce (*Picea abies*) synthesizes terpenoids (Schmidt et al., 2010). Despite having a sequenced genome (http://www.mimulusevolution.org/), however, the internal foliar secondary chemistry of yellow monkeyflower has remained unknown.

The presence of a diverse suite of PPGs in the foliage of *M. guttatus* (Phrymaceae) is not surprising, given its relatedness to the Scrophulariaceae (Beardsley and Olmstead, 2002). The Scrophulariaceae contains a rich diversity of PPGs and is one of the few plant families with mono-, di-, and trisaccharide PPGs (Mølgaard and Ravn, 1988). In addition, while PPGs occur in many other plant families, most notably in the Oleaceae and Orobanchaceae, Jimenez and Riguera (1994) list the Scrophulariaceae as having the greatest number of species with this class of secondary metabolites. With this in mind, we investigated yellow monkeyflower foliage for the presence of PPGs with the goal of incorporating knowledge of secondary metabolism into future studies of *Mimulus* evolution, genetics, and ecology.

Using NMR and liquid chromatography with UV and MS detection, we positively identified five PPGs from the leaves of yellow monkeyflower. Four of these compounds occur in various other species and one appears to be previously undescribed. We also present UV and high-resolution MS/MS data that putatively identify 11 additional compounds as PPGs.

2.1. NMR results

The ^{1}H and ^{13}C NMR chemical shifts of four of the compounds isolated from yellow monkeyflower closely match those of previously described phenylpropanoid glycosides (Fig. 1, Table 1, Suppl. Table 1, and Suppl. Figs. S1 and S2). The 1D-NMR spectra of compounds **1** and **2** correspond to those reported by several researchers for calceolarioside A and B, respectively (Chen et al., 2009; Damtoft and Jensen, 1994; Iossifova et al., 1999; Nicoletti et al., 1986; Shimomura et al., 1987). Both calceolarioside A and B are monosaccharide PPGs, which Nicoletti et al. (1986) initially isolated from *Calceolaria hypericina* (Calceolariaceae). These substances are positional isomers with the caffeoyl moiety at either C-4′ (calceolarioside A) or C-6′ (calceolarioside B) of glucose; apparent from the ^{1}H and ^{13}C chemical shift reversal we observed at those positions (Suppl. Table 1). HMBC correlations further confirmed the side group positions on compounds **1** and **2** (Suppl. Table 1). For instance, in calceolarioside A (**1**), HMBC correlations between H-4′ and C=O and the reciprocal correlations of H-1′ to C-8 and of the C-8 protons to C-1′ demonstrated the location of the caffeoyl and hydroxyphenylethyl groups, respectively. PPGs **3**–**5** displayed these same HMBC correlations, confirming that their hydroxycinnamic acid and hydroxyphenylethyl moieties have the same positions as those in calceolarioside A (**1**). Calceolarioside B (**2**), however, showed a correlation between both H-6′ protons and C=O, due to the alternative attachment of the caffeoyl group. Calceolarioside A corresponds to PPG 4 in our earlier work on variation of PPGs in natural populations of yellow monkeyflower (Holeski et al., 2013).

The 1D-NMR spectra of compound **3** matches that of conandroside (Jensen, 1996; Nonaka and Nishioka, 1977), a PPG originally isolated from *Conandron ramoidioides* (Nonaka and Nishioka, 1977). Unlike many other disaccharide PPGs, which have rhamnose as their side sugar, conandroside has a xylose bonded to C-3′ of the glucosyl moiety (Jimenez and Riguera, 1994; Mølgaard and Ravn, 1988). HMBC correlations of H-3′ to C-1″ and H-1″ to C-3′ further verified the specific connection of these two sugars (Suppl. Table 1).

NMR data identified compound **4** as verbascoside (Damtoft and Jensen, 1994; Nishimura et al., 1991; Owen et al., 2003), also known in much of the literature as acetoside, or less commonly kusaginin (Jimenez and Riguera, 1994; references therein). While overall very similar to conandroside (**3**), the ^{1}H NMR spectra of verbascoside (**4**) contained a doublet at δ_H 1.08 ppm, due to the three protons on the C-6″ methyl of the rhamnose side-sugar (Fig. 1). As with conandroside, HMBC data for verbascoside showed the same placement of this secondary sugar on the glucosyl moiety (Suppl. Table 1).

Compound **5** appears to be a new PPG (Søren Jensen, personal communication), which we have named mimuloside. Due to the presence of xylose as the secondary sugar, both the ^{1}H and ^{13}C NMR shifts of mimuloside closely match those of conandroside (**3**), except for additional distinctive shifts at δ_H 3.89 ppm and δ_C 56.44 ppm arising from the methoxy on the feruloyl moiety (Table 1, Suppl. Table 1; Chin et al., 2010; Froelich et al., 2008; Li et al., 2008). Furthermore, as with compounds **1**–**4**, the coupling constants of the α and β protons indicate a *trans*-configuration of the feruloyl group double bond (Nishimura et al., 1991). Thus, mimuloside is conandroside with ferulic instead of caffeic as its hydroxycinnamic acid.

The two-dimensional NMR results further confirmed the structure of mimuloside (**5**). HMBC correlations showed the same placement of both the hydroxycinnamic acid and hydroxyphenylethyl groups, as seen in **1**, **3**, and **4** (Table 1, Suppl. Table 1). A final key HMBC relationship included a correlation of the methoxy protons (δ_H 3.89) with C-3‴ of the feruloyl aromatic ring (δ_C 149.4), confirming mimuloside's hydroxycinnamic acid as ferulic (Li et al., 2008) and not the possible isoferulic acid seen on other PPGs, such as eukovoside (Stricher et al., 1982). We previously referred to mimuloside as PPG 5 in Holeski et al. (2013).

2.2. UHPLC-UV-TOF/MS results

The UV spectra of all compounds tested with UHPLC-UV-TOF/MS had very similar profiles with UV maxima near 327 nm (Table 2, Appendix 2), mostly due to UV activity of the hydroxycinnamic acid moiety (Pati et al., 2006; Li et al., 2005). These analyses also showed that individual plant samples differed greatly in the number of PPGs present and that the retention time of an authentic verbascoside standard closely matched that of compound **4** (Fig. 2, Appendix 2). Furthermore, extracted ion chromatograms for *m*/*z* 477 and 609 from TOF/MS analyses showed that some individual plant samples contained numerous isomers with these molecular weights (Fig. 2, Table 2).