Metabolite profiling reveals organ-specific flavone accumulation in *Scutellaria* and identifies a scutellarin isomer isoscutellarein 8-O-β-glucuronopyranoside

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Abstract
*Scutellaria* is a genus of plants containing multiple species with well-documented medicinal effects. *S. baicalensis* and *S. barbata* are among the best-studied *Scutellaria* species, and previous works have established flavones to be the primary source of their bioactivity. Recent genomic and biochemical studies with *S. baicalensis* and *S. barbata* have advanced our understanding of flavone biosynthesis in *Scutellaria*. However, as over several hundreds of *Scutellaria* species occur throughout the world, flavone biosynthesis in most species remains poorly understood. In this study, we analyzed organ-specific flavone profiles of seven *Scutellaria* species, including *S. baicalensis*, *S. barbata*, and two species native to the Americas (*S. wrightii* to Texas and *S. racemosa* to Central and South America). We found that the roots of almost all these species produce only 4′-deoxyflavones, while 4′-hydroxyflavones are accumulated exclusively in their aerial parts. On the other hand, *S. racemosa* and *S. wrightii* also accumulated high levels of 4′-deoxyflavones in their aerial parts, different from the flavone profiles of *S. baicalensis* and *S. barbata*. Furthermore, our metabolomics and NMR study identified the accumulation of isoscutellarein 8-O-β-glucuronopyranoside, a rare 4′-hydroxyflavone, in the stems and leaves of several *Scutellaria* species including *S. baicalensis* and *S. barbata*, but not in *S. racemosa* and *S. wrightii*. Distinctive organ-specific metabolite profiles among *Scutellaria* species indicate the selectivity and diverse physiological roles of flavones.

1 | INTRODUCTION

Medicinal plants have been used in the traditional medicines of indigenous populations for thousands of years. Due to this widespread usage, modern research techniques are being applied to identify the compounds responsible for these medicinal properties and to characterize their modes of action (Shang et al., 2010). A negative consequence of increased attention to and demand for medicinal plants is the endangerment of native plant populations resulting from overharvesting (Cole et al., 2007). Therefore, development of biotechnology-based mass production systems for these medicinal compounds is desirable. Development of effective biotechnology for chemical production requires an understanding of the biosynthesis of the compounds of interest. In this work, we analyze the levels of...
flavones in various organs of multiple species from the *Scutellaria* genus to better understand flavone biosynthesis in *Scutellaria*.

Part of the mint family Lamiaceae, *Scutellaria* is a genus of plants containing several hundred species with well-documented medicinal effects. Extracts from the aerial parts of *S. barbata* are commonly applied in Eastern medicines to treat swelling, inflammation, and cancer (Tao & Balunas, 2016). These activities, especially its anticancer effects, have drawn significant attention to *S. barbata*, and early phase clinical trials of aqueous extracts have demonstrated its selective cytotoxicity towards breast cancer cells (Chen et al., 2012). In addition, *S. barbata* extracts have exhibited remarkable activity towards multi-drug resistant strains of bacteria (Tsai et al., 2018). *S. baicalensis* is another species extensively applied in Eastern medicines, with extracts of its roots being prescribed to treat diarrhea, dysentery, hypertension, inflammation, and a variety of other diseases (Zhao, Tang, et al., 2019). Numerous clinical studies have demonstrated the neuroprotective, antibacterial, antitumor, antioxidant, and other beneficial health effects of these extracts (Saralamma et al., 2017; Tao et al., 2018; Zhu et al., 2016).

One class of bioactive compounds in *Scutellaria* is flavones (Karimov & Botirov, 2017; Zhao, Tang, et al., 2019). *Scutellaria* species produce two classes of flavones: 4′-hydroxyflavones and 4′-deoxyflavones (Figures 1 and S1). 4′-Hydroxyflavones, including apigenin and its derivatives, are relatively common across the plant kingdom, whereas 4′-deoxyflavones, which include chrysin and its derivatives, are relatively rare outside of *Scutellaria* (Kato et al., 1992; Rao et al., 2002; Rao et al., 2009). Recent works in *S. baicalensis* and *S. barbata* have identified multiple enzymes responsible for flavone biosynthesis in *Scutellaria* and have described the differential activity of specific enzymes towards either 4′-hydroxyflavones or 4′-deoxyflavones (Zhao et al., 2016, 2018; Zhao, Yang, et al., 2019). The enzyme selectivity leads to an organ-specific pattern of flavone accumulation. In this pattern, 4′-hydroxyflavones accumulate at higher concentrations in the aerial parts of the plant than in the roots, and the roots contain higher concentrations of 4′-deoxyflavones as compared to the aerial parts (Tao & Balunas, 2016; Xu et al., 2020).

Flavone profiles of *S. baicalensis* and *S. barbata* have been described, and their reference genomes have been established to further support the biosynthetic studies of flavones. However, due to the large number of uncharacterized species in the genus, it is unknown if the overall flavone pathway and the organ-specific accumulation patterns of *S. baicalensis* and *S. barbata* are well conserved across the genus. In this work, we aimed to expand the current knowledge of flavone diversity in *Scutellaria* by analyzing metabolite profiles of seven species. These species included two well-studied species, *S. baicalensis* and *S. barbata*, and two species native to warm climates, *S. racemosa* and *S. wrightii*. Furthermore, we selected three other *Scutellaria* species widely distributed in Europe, Asia, and North America, including *S. altissima*, *S. tournefortii*, and *S. parvula* (Hasaninejad et al., 2009; Shang et al., 2010; Sutter et al., 2011), respectively. During this analysis, we unexpectedly identified a 4′-hydroxyflavone which has not been included in the recent biosynthetic studies of *S. baicalensis*. We elucidated the structure of this 4′-hydroxyflavone and quantified its level in the seven species. Our results revealed diversity in site and type of flavone accumulated across the species we selected.

## Materials and Methods

### Plant growth conditions

Plants of seven *Scutellaria* species were grown from seed at the University of Florida (Gainesville, Florida, USA) in indoor, climate-controlled conditions at 21–23°C. Fluorescent lighting of intensity 140 μE m⁻² s⁻¹ was applied in a 16 h light/8 h dark cycle. Plants were watered every 5–8 days, and root, stem, and leaf tissue samples were collected from a field in Hattiesburg, Mississippi, USA, and grown in indoor, climate-controlled conditions at the University of Florida until seeds were ready to harvest. Seeds of *S. wrightii* were collected directly from mature plants grown in outdoor greenhouse conditions at Far South Wholesale Nursery (Austin, Texas, USA). Herbarium vouchers of all species were submitted to the University of Florida Herbarium, and voucher numbers are provided in Table S3.

### Flavone extraction and quantification

With High Performance Liquid Chromatography (HPLC), 15 flavones were quantified from root, stem, and leaf tissue samples of plants. The flavones quantified included seven 4′-hydroxyflavones, which were apigenin, apigenin-7-glucuronide (apigenin 7-G), scutellarein, scutellarin, hispidulin, hispidulose, and isoscutellarein-8-glucuronide (isoscutellarein 8-G). The remaining eight flavones were 4′-deoxyflavones, which were chrysin, chrysin-7-glucuronide (chrysin 7-G), baicalein, baicalin, oroxylin A, oroxyloside, wogonin, and wogonoside. The fresh weight of each tissue sample was determined with an analytical balance immediately after harvesting. Samples were frozen in liquid nitrogen and stored in −80°C until further analysis. An extraction solution of 50% HPLC grade methanol was added to ground samples so that the following ratio was achieved: 30 mg tissue/ml solvent. Samples were then sonicated for 1 h at room temperature. Following sonication, the extraction solution was withdrawn and further diluted with additional 50% methanol to achieve a final ratio of 30 mg tissue/ml solvent. To remove any remaining particulate, extractions were centrifuged at 15,000 rpm for 5 min and syringe filtered with a filter having a pore size of 0.45 μm.

Flavones were quantified in this final extraction with a Thermo Scientific (Massachusetts, USA) UltiMate 3000 HPLC system. Flavones were separated with an Acclaim RSLC 120 C18 column (2.2 μm, 3.0 × 100 mm) and eluted by a mixture of 1% formic acid
(A) and 100% acetonitrile (B). Following an 8-min equilibration with 5% B prior to injection, the following gradient was applied: 2 min, 25% B; 2 to 6 min, 25% B; 9 min, 50% B; 9 to 11 min, 50% B; 15 min, 95% B; and 15 to 23 min, 95% B. A flow rate of .5 ml/min was used and the column oven temperature set to 40°C. Peak areas were measured at wavelength 276 nm. Calibration mixes of .1, .5, 1, 5, 10, 25, 50, and 100 ppm were prepared with all 14 flavone standards except isocutellaren 8-G and used to convert peak areas to concentrations in ppm. Chemical standards used to prepare calibration mixes were purchased from ChemFaces (Wuhan, China) or MilliporeSigma (Massachusetts, USA) and dissolved in dimethylsulfoxide to generate stocks of 1,000, 2,000, or 4,000 ppm. These stocks were then diluted with 50% methanol and mixed to generate calibrations mixes of the varying concentrations. With the peak areas of these calibration mixes

**FIGURE 1** Proposed 4'-hydroxyflavone and 4'-deoxyflavone pathway. Structures of glycosylated flavones are not shown to save space but are included in Figure S1. Enzyme names in blue are specific isoforms that have been identified in *S. baicalensis*, and enzyme names in black are general names. Flavones that were quantified have names in bold and are numbered to match the labeling of Figure 2. Enzymes are phenylalanine ammonia lyase (SbPAL), cinnamate 4-hydroxylase (SbC4H), cinnamate-CoA ligase (SbCLL-7), 4-coumarate-CoA ligase (SbCLL-1), chalcone synthase (SbCHS-1), pinocembrin-chalcone synthase (SbCHS-2), chalcone isomerase (SbCHI), flavone synthase I (SbFNSI), flavone synthase II (SbFNSII), flavone 6-hydroxylase (SBF6H), flavone 8-hydroxylase (SbF8H), and 8-O-methyl transferase (Sb8-OMT).
and the molecular weight of each metabolite, flavone concentrations in μmol/g fresh weight were calculated. For relative concentration of isoscutellarein 8-G, only peak areas are reported.

Peaks in tissue sample chromatograms were identified based on their retention time and UV spectra (Figure S2) as compared with standards in the calibration mixes. Statistical testing was conducted by comparing the level of a flavone in the organ of a species to the level of the same flavone in the same organ of S. baicalensis. An unpaired Student’s t test with a significance cutoff of .05 was applied to identify values as statistically significant.

2.3 | LC-HRMS

LC-HRMS and HRMS/MS experiments were conducted on Thermo Scientific™ Q Exactive Focus mass spectrometer with Dionex™ Ultimate™ RSLC 3000 uHPLC system, equipped with H-ESI II probe on Ion Max API Source. Water with 1% formic acid (A) and acetonitrile with 1% formic acid (B) were used as the mobile phases to separate analytes on an Agilent Poroshell 120 EC-C18 column (2.7 μm, 3.0 x 50 mm). A typical LC program with a .5 ml/min flow rate included 10% B for 2 min, 10–95% B in 8.5 min, 95% B for 2.5 min, 95 to 10% B in .5 min, and re-equilibration in 2% B for 2 min. The eluents from the first 2 min and last 3 min were diverted to a waste bottle by a diverting valve. MS1 signals were acquired under the Full Eluents from the first 2 min and last 3 min were diverted to a waste

3 | RESULTS

3.1 | Organ-specific flavone diversity across seven Scutellaria species

We selected seven species of Scutellaria for organ-specific flavone profiling with High Performance Liquid Chromatography (HPLC). These species included S. altissima, S. baicalensis, S. barbata, S. parvula, S. racemosa, S. tournefortii, and S. wrightii. S. baicalensis and S. barbata have been used in East Asian medicines for thousands years. S. racemosa is native to South and Central America (Krings & Neal, 2001), and S. wrightii occurs in southwestern regions of North America, such as Texas (Nelson & Goetz, 2010). S. altissima, S. tournefortii, and S. parvula are widely distributed in Europe, Asia, and North America (Hasaninejad et al., 2009; Shang et al., 2010; Sutter et al., 2011), but their flavone profiles have not been studied extensively. We grew plants of each species from seed in climate-controlled conditions and harvested tissue samples from the roots, stems, and leaves of mature plants in biological triplicate. We then quantified concentrations of six 4’-hydroxyflavonones (1; apigenin, 2; apigenin 7-glucuronide (apigenin 7-G), 3; scutellarein, 4; scutellarin, 5; hispidulin, 6; hispiduloside) and eight 4’-deoxyflavonones (7; chrysin, 8; chrysin 7-glucuronide (chrysin 7-G), 9; baicalein, 10; baicalin, 11; oroxylin A, 12; oroxyloside, 13; wogonin, 14; wogonoside) in these samples (Figure 2; Table 1). S. baicalensis is one of the most studied and medicinally applied species, and we selected it as a reference for statistical testing in this work.

Our root-specific flavone profiling indicated that the 4’-deoxyflavone pathway appears to be very well-conserved across all seven species. We detected at least six of eight tested 4’-deoxyflavonones in the root of each species (Figure 2c; Table 1). Interestingly, although chrysin is proposed to serve as a precursor for all other 4’-deoxyflavonones, we found it only in the root of S. parvula, and its glycosylated form, chrysin 7-G, in the roots of three species, S. baicalensis, S. parvula, and S. tournefortii. On the other hand, we observed the accumulation of baicalein, baicalin, oroxylin A, and oroxyloside in the roots of all seven species (Figure 2c; Table 1). S. wrightii was the only species to accumulate a significantly greater amount (p < .05) of baicalin and oroxyloside in its roots than S. baicalensis. We also detected significantly elevated (p < .05) levels of baicalein and oroxylin A in the roots of S. wrightii as compared to S. baicalensis (Figures 2c and 3; Table 1). Chrysin can also be converted to wogonin and wogonoside through the reaction of SbFBH and SbB-OM (Figure 1). We detected both wogonin and wogonoside in the roots of all species except for S. parvula, where we did not detect wogonoside. S. tournefortii was the only species to
FIGURE 2  Metabolite data collected from the (a) leaves, (b) stems, and (c) roots of seven *Scutellaria* species via High Performance Liquid Chromatography (HPLC). Samples were taken in biological triplicate, and the average concentration of each metabolite calculated. Metabolites are numbered to match their order of occurrence in the flavone pathway, shown in Figure 1.
| Species       | Organ  | Apigenin | Apigenin 7-G | Scutellarein | Scutellarin | Isoscutellarein 8-G* | Hispidulin | Hispiduloside |
|--------------|--------|----------|-------------|--------------|-------------|----------------------|------------|--------------|
| S. baicalensis| Leaves | .19 ± .03 | .13 ± .01   | .27 ± .14    | .10 ± .10    | .10 ± .05            | n.d.       | n.d.         |
| S. baicalensis| Stems  | .12 ± .12 | .22 ± .01   | 1.04 ± .43   | 1.76 ± .28   | 2.17 ± .60           | .03 ± .02  | n.d.         |
| S. baicalensis| Roots  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. altissima  | Leaves | .14 ± .08 | .03 ± 1.5   | .65 ± .28    | 4.17 ± 1.22  | n.d.                 | n.d.       | n.d.         |
| S. altissima  | Stems  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. altissima  | Roots  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. barbata    | Leaves | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. barbata    | Stems  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. barbata    | Roots  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. parvula    | Leaves | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. parvula    | Stems  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. parvula    | Roots  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. racemosa   | Leaves | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. racemosa   | Stems  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. racemosa   | Roots  | n.d.     | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. tournefortii| Leaves| n.d.    | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. tournefortii| Stems | n.d.    | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. tournefortii| Roots | n.d.    | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. wrightii   | Leaves | n.d.    | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. wrightii   | Stems  | n.d.    | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |
| S. wrightii   | Roots  | n.d.    | n.d.        | n.d.         | n.d.        | n.d.                 | n.d.       | n.d.         |

(Species)
### Table 1

| Species          | Organs | Apigenin 7-G | Apigenin | Scutellaran 8-G | Isocoumarin 8-G | Hipsiduloid | Hipsidolin | Hipsidulide |
|------------------|--------|--------------|----------|-----------------|-----------------|-------------|------------|------------|
| S. barbata       | Roots  | 0.40 ± 0.08  | n.d.     | 0.12 ± 0.03     | 0.18 ± 0.04     | 2.18 ± 0.33 | 56.62 ± 1.10|
| S. barbata       | Leaves | 0.12 ± 0.01  | n.d.     | 0.24 ± 0.22     | 0.63 ± 2.13     | 5.35 ± 0.94 | 7.96 ± 3.31|
| S. barbata       | Stems  | 0.03 ± 0.00  | n.d.     | 0.34 ± 0.08     | 0.59 ± 1.09     | 0.35 ± 0.53 | 0.32 ± 0.93|
| S. racemosa      | Roots  | 0.40 ± 0.08  | n.d.     | 0.12 ± 0.03     | 0.18 ± 0.04     | 2.18 ± 0.33 | 56.62 ± 1.10|
| S. racemosa      | Leaves | 0.12 ± 0.01  | n.d.     | 0.24 ± 0.22     | 0.63 ± 2.13     | 5.35 ± 0.94 | 7.96 ± 3.31|
| S. racemosa      | Stems  | 0.03 ± 0.00  | n.d.     | 0.34 ± 0.08     | 0.59 ± 1.09     | 0.35 ± 0.53 | 0.32 ± 0.93|
| S. tournefortii  | Roots  | 0.40 ± 0.08  | n.d.     | 0.12 ± 0.03     | 0.18 ± 0.04     | 2.18 ± 0.33 | 56.62 ± 1.10|
| S. tournefortii  | Leaves | 0.12 ± 0.01  | n.d.     | 0.24 ± 0.22     | 0.63 ± 2.13     | 5.35 ± 0.94 | 7.96 ± 3.31|
| S. tournefortii  | Stems  | 0.03 ± 0.00  | n.d.     | 0.34 ± 0.08     | 0.59 ± 1.09     | 0.35 ± 0.53 | 0.32 ± 0.93|
| S. wrightii      | Roots  | 0.40 ± 0.08  | n.d.     | 0.12 ± 0.03     | 0.18 ± 0.04     | 2.18 ± 0.33 | 56.62 ± 1.10|
| S. wrightii      | Leaves | 0.12 ± 0.01  | n.d.     | 0.24 ± 0.22     | 0.63 ± 2.13     | 5.35 ± 0.94 | 7.96 ± 3.31|
| S. wrightii      | Stems  | 0.03 ± 0.00  | n.d.     | 0.34 ± 0.08     | 0.59 ± 1.09     | 0.35 ± 0.53 | 0.32 ± 0.93|

**Note:** Units for all flavones are μmol/g fresh weight, except for isocoumarin 8-G, which has units of peak area. Data are presented as mean ± standard error, as calculated from samples taken in biological triplicate.

**Abbreviations:** HPLC, High Performance Liquid Chromatography; n.d., not detected.*

![Image](https://example.com/image.png)

**3.2 The structural elucidation of a new scutellarin isomer**

During our metabolite analysis, we detected multiple new metabolites which we were unable to unambiguously assign their identities. Of these unknown metabolites, one drew our interest because of its pattern of accumulation across the tissue samples we collected (Figure 4). In our HPLC chromatograms, we detected the peak corresponding to this metabolite in the aerial parts of S. baicalensis and S. barbata, but not in S. racemosa. The peak was absent in root chromatograms collected from all seven species. The aerial specificity of this unknown metabolite led us to hypothesize that it was a 4′-hydroxyflavone. To elucidate its structure, we analyzed the
unknown metabolite from our *S. barbata* leaf extracts by the liquid chromatography-high resolution mass spectrometry (LC-HRMS). Interestingly, its molecular weight was identical to scutellarin ([M + H]⁺ m/z 463.0866, calculated for C₂₁H₁₉O₁₂⁺, 463.0871), but they were eluted with different retention times (tᵣ = 6.28 min for scutellarin vs. 6.94 min for the unknown compound) (Figure 5a). Furthermore, they gave rise to the same major MS/MS fragment, suggesting them to be two isomers (Figure S3).

To further elucidate the structure of this compound, we performed 1D and 2D NMR analysis (Figures S4–S6). Comparison of its 

- **Discussion**

From our analysis of organ-specific flavone diversity, we determined profiles for *S. baicalensis* and *S. barbata*, which matched closely with previous publications (Xu et al., 2020; Zhao et al., 2016). In these flavone profiles, high concentrations of 4'-deoxyflavones accumulated in the roots, and much lower concentrations of 4'-deoxyflavones and 4'-hydroxyflavones accumulated in the stems and leaves (Figure 2; Table 1). As described by Zhao et al. (2016), the root-favored accumulation of 4'-deoxyflavones by *S. baicalensis* is due to root-specific overexpression of several enzyme isoforms with activity exclusively, or near exclusively in 4'-deoxyflavone biosynthesis (Zhao et al., 2016). In contrast to the pattern we observed in *S. baicalensis* and *S. barbata*, we identified that *S. racemosa* and *S. wightii* accumulated no isoscutellarein 8-G in their aerial parts. It is noteworthy that these three species accumulated oroxylin A and its glycoside in their aerial parts (Figure 3).
isoforms is not perfectly root-specific and some enzymes having activities towards 4\(^\circ\)-deoxyflavone precursors such as SbCLL-7 and SbCHS-2 may be active in both roots and aerial parts at least under our growth conditions. It is also possible that some fraction of 4\(^\circ\)-deoxyflavones are synthesized in the roots and then transported to the aerial parts. The fact that 4\(^\circ\)-hydroxyflavones were not detected in roots of most species indicates the selectivity of enzymes towards either 4\(^\circ\)-deoxyflavones or 4\(^\circ\)-hydroxyflavones (or their respective precursors), as well as organ-specific regulation of biosynthetic gene expression.

We found that S. racemosa accumulates the highest concentrations of oroxylin A, and its 7-glucuronide, oroxyloside, in its leaves, among all organs of all species (Figure 3; Table 1). S. wrightii also accumulated notable amounts of oroxylin A and oroxyloside in its stem, but not in its leaves. Oxyroxylin A is a 4\(^\circ\)-deoxyflavone which has been demonstrated to exhibit memory enhancement and neuroprotective...
effects in rat models (Jeon et al., 2011, 2012). The most likely route for oroxylin A biosynthesis is the methylation of baicalein at its 6-OH group (Figure 1) (Elkin et al., 2018). Although previous works have identified a variety of O-methyltransferases (OMTs) in plants, the enzymes with high specificity for the 6-OH group in flavonoids are rare, as this reaction is biochemically unfavorable (Zhang et al., 2016). Work in sweet basil (Ocimum basilicum), a species also in the Lamiaceae family with Scutellaria, identified a methyltransferase capable of specific methylation of the 6-OH group of scutellarein (Berim et al., 2012). Scutellarein is a 4′-hydroxyflavone identical in structure to baicalein apart from its 4′-OH group. To ensure the proper orientation of its substrate, and thus its regioselectivity, the O. basilicum OMT uses a threonine residue to form a hydrogen bond with the 4′-OH group of scutellarein. However, as baicalein has no 4′-OH group, it would be impossible for a regioselective OMT in S. racemosa or S. wrightii to rely on this interaction during the methylation of baicalein. Research by Zhang et al. (2016) in a liverwort species (Plagiochasma appendiculatum) identified a methyltransferase (PaF6OMT) that is capable of methylation of the 6-OH group in baicalein. As this OMT has not yet been structurally characterized, how it achieves its specificity remains unknown. Future work in S. racemosa and S. wrightii should be directed towards characterizing its biosynthesis of oroxylin A, with specific attention paid to the potential specialization of OMTs in the pathway. Overall, S. racemosa and S. wrightii are promising targets for biotechnology improvement due to the significant bioactive effects of oroxylin A and oroxyloside. Considering that both species occur in warm area (Texas and South America) (Krings & Neal, 2001; Nelson & Goetze, 2010), accumulation of oroxylin A and oroxyloside in these species may indicate the physiological relevance of oroxylin A and oroxyloside in these species.

Isoscutellarein 8-G was first detected in the liverwort species Marchantia berteroana (Markham & Porter, 1975). Following this initial report, Miyaichi, Imoto, et al. (1988) and Miyaichi, Kizu, et al. (1988) detected the flavone in the aerial parts of S. indica and S. baicalensis. Aside from these works, few other studies have reported isoscutellarein 8-G in Scutellaria, though several have detected its aglycone and 7-O-glycosylated forms (Karimov & Botirov, 2017). This rarity in detection may be a result of its low abundance relative to other glycosylated flavones in Scutellaria. A potential reason for this low abundance is its unique glycosylation at the 8-O position. Flavone 7-O glycosylation is more common in Scutellaria due to the presence of a hydroxyl group at the 7-O position in all flavones synthesized via the core flavone pathway (Figure 1). On the other hand, 8-O glycosylation first requires the activity of an 8-hydroxylase to add the free hydroxyl group to which the carbohydrate will be attached. As the purpose of glycosylation is typically to increase the stability of the flavone for long term storage (Slámová et al., 2018), it is possible that 8-O glycosylation provides slightly greater stability as compared to 7-O glycosylation. Therefore, it would be preferable to glycosylate isoscutellarein at the 8-O position. Flavone 7-O glycosylation is more common in Scutellaria due to the presence of a hydroxyl group at the 7-O position in all flavones synthesized via the core flavone pathway (Figure 1). On the other hand, 8-O glycosylation first requires the activity of an 8-hydroxylase to add the free hydroxyl group to which the carbohydrate will be attached. As the purpose of glycosylation is typically to increase the stability of the flavone for long term storage (Slámová et al., 2018), it is possible that 8-O glycosylation provides slightly greater stability as compared to 7-O glycosylation. Therefore, it would be preferable to glycosylate isoscutellarein at the 8-O position, even though a free hydroxyl group is also present at the 7-O position. Several species may have evolved regioselective glycosyltransferase enzymes for this purpose. Researchers working with a glycosyltransferase from Bacillus cereus demonstrated that a single amino acid substitution could alter the primary site of quercetin glycosylation with high specificity (Chiu et al., 2016). Perhaps, a similar mutation occurs in several Scutellaria species to allow the biosynthesis of isoscutellarein 8-G. Alternatively, it is possible that the glycosyltransferase enzymes of these species which accumulate isoscutellarein 8-G have less strict regioselectivity, and are capable of glycosylation at both 7-G and 8-G positions. Quantification of isoscutellarein 7-G alongside isoscutellarein 8-G would
provide valuable insight regarding these theories. Based on current understanding of flavone biosynthesis, we propose a possible route of isoscetullarein and isoscetullarein 8-G production from apigenin (Figure S7). Further organ-specific transcriptome study is required to identify enzymes responsible for isoscetullarein and isoscetullarein 8-G production.

Our quantification of isoscetullarein 8-G across the seven Scutellaria species we analyzed revealed an intriguing pattern. Isoscetullarein 8-G was entirely absent in the species of S. parvula, S. racemosa, and S. wrightii, all of which accumulate high concentrations of 4’-deoxyflavones such as oroxylin A and oroxyloside in their aerial parts. This specific example is representative of a broader pattern—species with high accumulation of 4’-deoxyflavones in their aerial parts accumulated low concentrations of 4’-hydroxyflavones. This substitution of 4’-hydroxyflavones with 4’-deoxyflavones potentially indicates an evolution to utilize 4’-deoxyflavones to fulfill the physiological roles which 4’-hydroxyflavones do in other species. Works in species outside of Scutellaria have demonstrated the anti-herbivory effects of several 4’-hydroxyflavones we quantified here (Gallon et al., 2019; Sosa et al., 2004). However, little is known about the physiological role that 4’-deoxyflavones play in plants. Further research should be devoted to exploring the role of 4’-deoxyflavones in plant growth and stress response to better understand the evolutionary advantage their biosynthesis and accumulation offers.

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AUTHOR CONTRIBUTIONS
B.C.A., Y.D., and J.K. designed the research project; B.C.A., D.L., G.M.R., and Y.S. performed the experiments and analyzed the data; B.C.A., Y.D., and J.K. wrote the manuscript.

CONFLICT OF INTEREST
The authors declare no competing interest.

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