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Foliar phenolic glycosides from *Populus fremontii*, *Populus angustifolia*, and their hybrids

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Abstract

Salicortin (**1**) and HCH-salicortin (**2**) were isolated and identified from the foliage of *Populus fremontii* and its F_1 hybrids with *Populus angustifolia*. Salicortin, but not HCH-salicortin, also occurred in *P. angustifolia* and complex backcrosses to *angustifolia*. Concentrations ranged from 0 to 17.5% dry weight for salicortin and 0 to 5.9% dry weight for HCH-salicortin. HCH-salicortin may possess potent anti-herbivore activity as it contains two of the hydroxycyclohexen-on-oyl moieties known to confer such activity to salicortin. Further, this compound may be a useful chemotaxonomic character within the genus *Populus*, since it appears to occur in section *Aigeiros* but not in section *Tacamahaca*.

Keywords: *Populus fremontii*; *Populus angustifolia*; Salicaceae; Ecological biochemistry; Salicortin; HCH-salicortin; Anti-herbivore compounds

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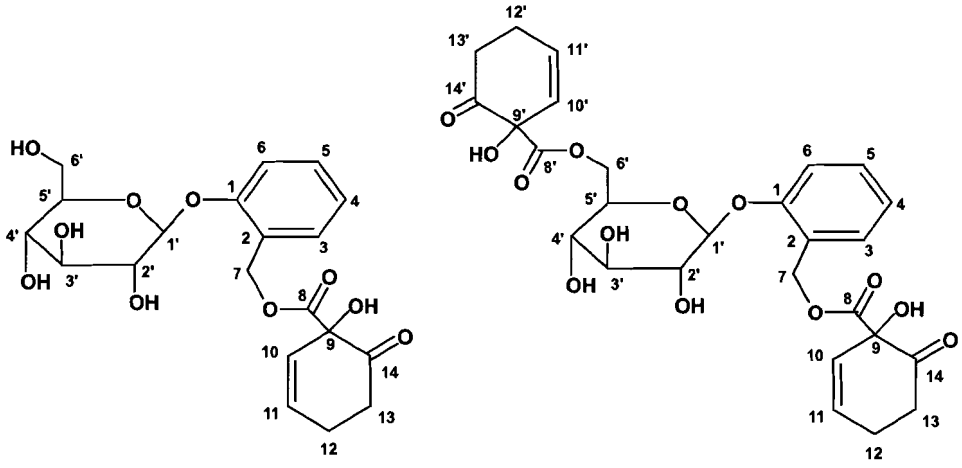
1. Introduction

Phenolic glycosides are important secondary compounds in trees of the genus *Populus* as well as other plant species in the family Salicaceae (Thieme and Benecke, 1971; Palo, 1984; Julkunen-Tiitto, 1986; Julkunen-Tiitto, 1989). These salicylate compounds mediate plant resistance to both insect and mammalian herbivores, and anti-herbivore activity is often associated with the core structure of salicortin (**1**), especially the hydroxycyclohexen-on-oyl moiety attached to C-7. Salicortin and tremulacin both function as chemical defenses against generalist herbivores in quaking aspen (*Populus tremuloides*) (Lindroth and Hwang, 1996). Compounds with similar structures are likely to have similar anti-herbivore activity, thus we investigated the distribution and concentrations of salicylate glycosides in leaves of Fremont cottonwood (*Populus fremontii* L.), narrowleaf cottonwood (*Populus angustifolia* James), their F_1 hybrids, and complex backcrosses to narrowleaf cottonwood. The ecology of this *Populus* system has been well characterized: various studies have examined habitat selection (Whitham, 1980), trophic interactions (Floate et al., 1993; Wimp and Whitham, 2001), community organization (Dickson and Whitham, 1996; Whitham et al., 2003), and ecosystem function (Driebe and Whitham, 2000). Phenolic glycosides mediate some ecological interactions in this cottonwood system (Kearsley and Whitham, 1992; Martinsen et al., 1998), but their precise distribution, roles and identities have been poorly understood. We found both salicortin (**1**) and a related compound, HCH-salicortin (**2**) (Fig. 1).



1 **2**

Fig. 1. Structures of salicortin (**1**) and HCH-salicortin (**2**).

2. Methods

2.1. Plant material

Leaves of Fremont cottonwood (*P. fremontii*), narrowleaf cottonwood (*P. angustifolia*), and their hybrids were collected in September 2000 from a garden at the Ogden Nature Center, Ogden, Utah, USA. Samples consisted of 15–25 leaves from position 5 on a shoot collected from branches in the lower canopy, with a shoot considered the current year's growth. Leaves were flash frozen between blocks of dry ice and kept frozen until lyophilization (freeze drying). Lyophilized leaves were ground to pass the 40 mesh screen of a Wiley Mill and stored at −20 °C until use. These samples were used in the initial screening for phenolic glycosides as well as quantification. Fremont cottonwood leaves for extraction and structural elucidation of phenolic glycosides were collected from a single tree ("XH-11") by the same procedure from a field site ("Hull") near Ogden, Utah in June and July, 2002, but leaves were taken from all positions on a shoot. Tree species and hybrid identities were confirmed using RFLP markers developed for this hybrid swarm (Martinsen et al., 2001). Introgression is unidirectional in this system: the F_1 hybrids backcross only to narrowleaf cottonwood. All trees used in the study have been genetically fingerprinted, tagged, mapped, and located by GPS, and can thus be considered living vouchers.

2.2. General procedures

High performance thin layer chromatography (HPTLC) was performed as described by Lindroth et al. (1993). In brief, ca. 25 mg aliquots of freeze-dried foliage were extracted in ice cold MeOH with sonication, centrifuged, and applied in duplicate to silica gel HPTLC plates. Plates were developed in CH_2Cl_2:MeOH:THF (30:5:5), and the chromatograms were analyzed with a Camag TLC scanner and associated software (Camag Scientific, NC, USA). Qualitative thin layer chromatography used in conjunction with the preparative chromatography was done on silica gel plates developed in CH_2Cl_2:MeOH (18:3) and visualized with iodine.

2.3. Initial screening of leaf samples

Leaf samples from 43 trees in the Ogden Nature Center common garden were screened for the presence of phenolic glycosides by HPTLC using salicin, salicortin (**1**), tremuloidin and tremulacin purified from aspen (Lindroth et al., 1987) as standards. Identification of phenolic glycosides contained in the samples was based on comparison of chromatographic and UV spectral properties with those of the standards.

2.4. Isolation procedures

2.4.1. Extraction of salicortin and HCH-salicortin from bulk material

Phenolic glycosides were extracted and isolated by a modification of the procedure of Lindroth et al. (1987). Freeze-dried Fremont foliage (120 g) was extracted sequentially with 1.0, 1.0, and 0.5 l of Me_2CO (neat at 0 °C), and the combined Me_2CO extracts were evaporated under vacuum and lyophilized. This residue (ca. 17 g or 14%) was dissolved in 70% MeOH (aq) at 0 °C, filtered, and partitioned first against 3:1 hexane:$CHCl_3$ (5×), retaining the MeOH phase, and then extracted with $CHCl_3$ (3×). The combined $CHCl_3$ extracts were evaporated under vacuum and lyophilized, yielding ca. 7.9 g (6.6%).

Aliquots (1.0 g) of the partitioned $CHCl_3$ extract were loaded onto a 5.0 cm diameter flash chromatography column that contained silica gel (Sigma S-0507; Sigma, St. Louis, MO, USA) and 10% MeOH in $MeCl_2$. The column was eluted with 10% MeOH in $MeCl_2$, and 20 ml fractions were collected after allowing ca. 300 ml of solvent to run through the column. Based on qualitative TLC, two sets of fractions (ca. 11–24 and 32–45) were combined, and each isolate was evaporated under vacuum. Typical yields were 330 mg of crude **2** and 270 mg of crude **1**. Each isolate was rechromatographed by loading 200 mg onto a column containing silica gel and 8% MeOH in $MeCl_2$. Appropriate fractions were combined based on qualitative TLC, evaporated under vacuum, and lyophilized. Typical yields per column were 150 mg for **1** and 120 mg for **2**. This purified material was used for structural elucidation.

2.4.2. Characterization of isolates by spectral analyses

Salicortin (**1**) and HCH-salicortin (**2**) were characterized by electrospray ionization mass spectrometry and high resolution NMR spectroscopy. Mass spectra were obtained on methanol solutions of the compounds using a Micromass LCT mass spectrometer. NMR spectra were obtained on solutions of the compounds in methanol-d_4 using a Varian Mercury 300 NMR system with a broadband tunable probe. ^1H and ^{13}C NMR spectra were obtained at 300 MHz and 75 MHz, respectively. Multiplicities of ^{13}C NMR peaks were determined using DEPT. Two dimensional ^1H–^1H and ^1H–^{13}C correlations were made using standard pulse sequence experiments (COSY, HETCOR, HMBC, HMQC).

2.5. Quantification of salicortin and HCH-salicortin

Both compounds were quantified by HPTLC of the samples used for initial screening, employing the purified compounds as standards.

3. Results and discussion

Initial screens indicated the presence of salicortin in all samples and an unidentified phenolic glycoside in Fremont and F_1 hybrid samples. Trace amounts of salicin (<0.5%) were found in 28 of the 43 samples. These data indicated that salicortin and the unidentified phenolic glycoside (**2**) were present at concentrations known from other *Populus* species to affect herbivore behavior and performance.

The unidentified compound was characterized by mass spectrometry and NMR spectroscopy. The mass spectrum of the compound, using electrospray ionization, exhibited a peak at *m/z* 585 corresponding to $[M + Na^+]$, indicating a molecular formula of $C_{27}H_{30}O_{13}$. The compound was identified as HCH-salicortin (**2**) by comparison of its ^{13}C and ^1H NMR spectral data to those previously reported for this compound (Picard et al., 1994). Further confirmation of the structure of HCH-salicortin was made using DEPT and 2-D NMR experiments. The NMR data and assignments are summarized in Table 1.

The ^{13}C NMR spectrum of HCH-salicortin is very similar to that of salicortin except the signals corresponding to carbon positions 8 through 14 in HCH-salicortin appear as "twin" signals with chemical shift differences within each pair ranging from 0.02 ppm to 0.29 ppm. This pattern is consistent with the presence of two hydroxycyclohexen-on-oyl moieties in slightly different chemical shift environments. Comparison of the ^{13}C NMR signal pattern of the glycoside carbons (C-1′–C-6′) with those previously reported for a variety of different phenolic glycosides indicated esterification at the C-6′ position of the glucose moiety (Dommisse et al., 1986). Confirmation of the positions of the two hydroxycyclohexen-on-oyl moieties at C-7

Table 1
^1H and ^{13}C NMR data and assignments for HCH-salicortin

^{13}C NMR δ (ppm)	Position[a]	^1H NMR δ (ppm)	J_{H-H} (Hz)
157.00	1	–	
126.83	2	–	
130.80	3	7.0–7.7 (4H, m)	
123.98	4		
131.34	5		
117.36	6		
64.50	7	5.30 (2H, a,b quartet)	$J_{7a-7b} = 12.0$
102.90	1′	4.92 (1H, m)	
74.90	2′	3.47 (2H, m)	
77.79	3′		
71.50	4′	3.30 (1H, m)	
75.41	5′	3.63 (1H, ddd)	$J_{5'-4'} = 9.8$
66.26	6′	4.24 (H_a, dd) 4.56 (H_b, dd)	$J_{6'a-6'b} = 11.8$, $J_{6'a-5'} = 6.5$, $J_{6'b-5'} = 2.1$
171.52	8′	–	
171.62	8	–	
79.25, 79.36	9,9′	–	
129.41, 129.43	10,10′	5.69 (1H, dt), 5.75 (1H, dt)	$J_{10-11} = J_{10'-11'} = 9.8$, $J_{10-12} = J_{10'-12'} = 1.7$
133.45, 133.51	11,11′	6.07 (1H, dt), 6.14 (1H, dt)	$J_{11-12} = J_{11'-12'} = 3.8$
27.34, 27.36	12,12′	2.35–2.95 (8H, m)	
36.91, 36.95	13,13′		
207.25, 207.54	14,14′	–	

[a] Assignments in the same row may be reversed.

Table 2
Quantities of salicortin and HCH-salicortin in the foliage of Fremont and narrowleaf cottonwoods and their hybrids

Cross	N	Salicortin				HCH-salicortin			
		Mean	SD	Min	Max	Mean	SD	Min	Max
Fremont	15	4.9	2.21	1.0	9.0	2.1	1.72	1.0	5.9
F1 hybrid	6	7.2	4.25	3.8	15.8	3.3	1.15	1.9	3.6
Backcross hybrid	11	6.2	6.22	0.0	17.3	0.0	0.00	0.0	0.0
Narrowleaf	21	8.8	4.14	0.0	17.5	0.0	0.00	0.0	0.0

All quantities are given as percent of dry weight.

and C-6′ was obtained from a ^{1}H–^{13}C HMBC NMR experiment that clearly showed three-bond ^{1}H–^{13}C interactions at these positions. (C-8/H-7a, H-7b: δ 171.62 ppm/5.31 ppm) C-8′/H-6a, H-6b: δ 171.52 ppm/4.24, 4.56 ppm).

Based on quantification of salicortin and HCH-salicortin with HPTLC as detailed in Table 2, these compounds occur in concentrations that may have anti-herbivore activity (Lindroth and Hwang, 1996). In addition to the differences *among* these species and their hybrids, concentrations of salicortin and HCH-salicortin also vary widely *within* some categories (e.g. salicortin in narrowleaf cottonwood). Given the presence of the hydroxycyclohexen-on-oyl moiety in the structure of HCH-salicortin, it is likely that this compound will have potent anti-herbivore effects (Scriber et al., 1991; Nichols-Orians et al., 1992).

HCH-salicortin may also be useful as a chemotaxonomic marker for the section *Aigeiros* of the genus *Populus*. It occurs in *P. fremontii* (section *Aigeiros*) as well as in the F_1 hybrids of *P. fremontii* and *P. angustifolia*, but not in pure *P. angustifolia* (section *Tacamahaca*). HCH-salicortin has also been isolated from eastern cottonwood (*Populus deltoides*, section *Aigeiros*; (Picard et al., 1994)). Further, hybridization between sections *Aigeiros* (2 species) and *Tacamahaca* (3 species) in North America has been studied (Eckenwalder, 1984), and five of the six possible different hybrids have been recorded from nature. The presence of HCH-salicortin may prove to be a useful character to confirm the *Aigeiros* parentage of suspected hybrids.

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