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2′-(*Z*)-Cinnamoylsalicortin: A novel salicinoid isolated from *Populus tremula*

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ABSTRACT

Using a combination of NMR and mass spectroscopic techniques, we have isolated a new salicinoid from the foliage of European aspen (*Populus tremula*) and identified it as 2′-(*Z*)-cinnamoylsalicortin. The relatively high amounts in foliage and the similarity in structure to bioactive salicinoids isolated from other salicaceous trees indicates that this compound may have implications for the study of *P. tremula*-herbivore interactions.

1. Introduction

Salicinoids, also known as phenolic glycosides (Lindroth et al., 1987) or salicylates (Haikio et al., 2009), are the primary anti-herbivore secondary compounds of species in the Salicaceae (Boeckler et al., 2011). Several of these compounds have proven bioactive properties against insect (Donaldson and Lindroth, 2007) and mammalian (Wooley et al., 2008) herbivores and studies with the North American aspen, *Populus tremuloides*, suggest that their toxicity increases with greater structural complexity (Philippe and Bohlmann, 2007). While no clear mechanism currently exists, the toxicity of more complex salicinoids is attributed to the hydro-cyclohexen-on-oyl (HCH) group, which is thought to be synergized by other co-occurring aromatic moieties (Lindroth et al., 1988).

The most common and usually most abundant salicinoid is salicortin (Fig. 1: **1**); however, many species also produce more complex salicinoids, many of which are salicortin derivatives with additional moieties often attached to the 2′ or 6′ position of the glucose unit (Boeckler et al., 2011). 2′-Cinnamoylsalicortin (**2**) represents an example of these more complex salicinoids. Nichols-Orians et al. (1992) originally isolated and identified **2** from the leaves of silky willow (*Salix sericea*). The foliar concentration of **2** varies with clone identity and together with salicortin it is considered the most bioactive compound in this willow species (Orians et al., 2000). Abreu et al. (2011) recently reported **2** in European aspen (*Populus tremula* L., Salicaceae), also with highly variable levels among Swedish clones and in similar or greater concentrations than those reported for *S. sericea* (Orians et al., 2000). Nichols-Orians et al. (1992) suspected the presence of a regioisomer of **2** in *S. sericea*, based upon ^{13}C NMR results, and Abreu et al. (2011) found a second compound with the same molecular weight at an earlier UPLC–MS retention time in *P. tremula*; however, neither determined the structure of the second isomer. It is important to distinguish isomers in studies that combine plant genetic structure and associations with herbivores and other antagonists (Bernhardsson et al., 2013; Robinson et al., 2012), since they can differ in their toxicity to herbivores. For instance, *trans*- fused-ring sesquiterpene lactones from the herb *Xanthium strumarium* deterred an orthopteran herbivore more effectively than their *cis*- fused-ring isomers (Ahern and Whitney, 2013).

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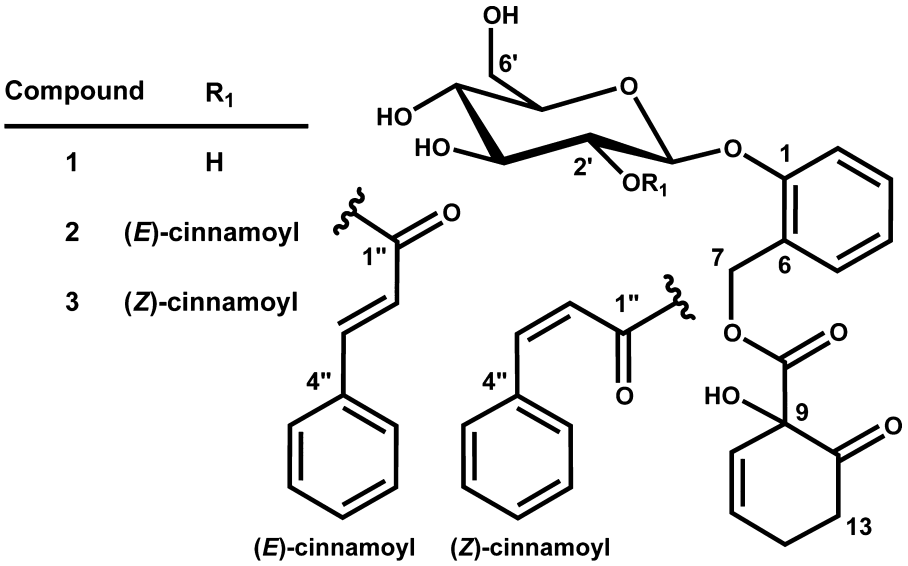

Fig. 1. Structures of salicortin (**1**), 2′-(*E*)-cinnamoylsalicortin (**2**), and 2′-(*Z*)-cinnamoylsalicortin (**3**) and NMR designations for **2** and **3**.

We now report the structure of the previously undescribed salicinoid, 2′-(*Z*)-cinnamoylsalicortin (**3**), isolated from the foliage of *P. tremula*. The characterization of this novel salicinoid adds to the rich chemical diversity of salicinoids in *P. tremula* and in the Salicaceae. In addition, given its complex structure and similarity to known bioactive compounds, it may help mediate plant-herbivore interactions in this system.

2. Results and discussion

Abreu et al. (2011) described several novel salicinoids from the foliage of *P. tremula* and noted the presence of specific salicinoid chemotypes within *P. tremula*, including one that contained substantial amounts of **2**. A detailed look at the chemistry of additional *P. tremula* genotypes from the Swedish aspen collection (SwAsp; Luquez et al., 2008) has led to the discovery of even more salicinoids new to European aspen, including **3** (Keefover-Ring et al., unpublished data). These preliminary analyses showed that the levels of **3** approach those of the known isomer **2**, adding to the chemical diversity and possible toxicity of this species.

2.1. NMR results

Due to overall structural similarity (Fig. 1), most of the one- and two-dimensional ^{1}H and ^{13}C NMR values from **3** (Table 1; Supplementary Fig. 1) closely matched those of **2** (Table 2). The ^{13}C chemical shifts of the 2′ glucose carbon for both compounds were much further downfield than salicinoids unsubstituted at this site (Dommisse et al., 1986), indicating the presence of a 2′-cinnamoyl moiety on both. Results from the HMBC experiments also confirmed the cinnamoyl group at the 2′ position, shown by a clear correlation between H-2′ to C-1″ for both **2** and **3**. Differences in ^{1}H and ^{13}C NMR shifts for the double bond in the cinnamoyl substituent at position 2″ and 3″ and the difference in the coupling constant between compound **2** and **3** showed that the respective isomers had either an (*E*)- or (*Z*)-cinnamoyl. The coupling constants for $J_{2''\text{-}3''}$ were 16.0 Hz for compound **2** and 12.5 for **3**, which correspond to (*E*)- and (*Z*)-cinnamoyl, respectively. In addition, we observed a distinct ROESY cross peak between H-2″ and H-3″ for compound **3**, but not for **2**, which verifies the (*Z*)-conformation of the cinnamoyl group in **3** (Supplementary Fig. 2).

2.2. LC–MS and tandem mass spectroscopy results

Initial UPLC–TOFMS analysis showed that both **2** and **3** have the same molecular weight with both compounds appearing mostly with a formate adduct (599 *m*/*z*) under the conditions tested. In addition, the second UV maxima for both of the isomers were similar to one another and higher than most of the salicinoids found by Abreu et al. (2011) (Table 3). Analysis of the *P. tremula* foliage used for compound isolation showed that *in planta* the isomers exist in a ratio of 59 (*E*): 41 (*Z*) with a combined concentration of 7.6 mg g^{-1} DW. Overall, these two compounds represented 21.5% of the total of 19 salicinoids tested for, the third highest behind salicortin at 39.5%. These values are comparable with other Swedish *P. tremula* clones that contain 2′-cinnamoyl-salicortin (Keefover-Ring et al. unpublished data).

High-resolution tandem mass spectroscopy analysis revealed a fragmentation pattern for **3** that was very similar to that of **2** from the current isolations and from Abreu et al. (2011) (Table 3). For both compounds, the most abundant fragment was 405 *m*/*z*, resulting from the loss of either a (*E*)- or (*Z*)-cinnamoyl moiety from the 2′ glucose carbon. The two isomers shared other prominent fragments, including 429 and 447 *m*/*z* due to loss of the salicylyl group and two subsequent rearrangements, 415 *m*/*z* from the cleavage of the HCH moiety, and 285 *m*/*z* due to loss of both the cinnamoyl and HCH groups. Finally, calculated exact masses and formulas for **2** and **3** closely matched one another and theoretical values (Table 3).

3. Experimental

3.1. Plant material

We collected *P. tremula* foliage on July 15, 2011 from a single ~3 m high aspen tree designated clone 23 from the SwAsp collection growing at the Skogforsk research station near Sävar, Sweden. Previous analysis by Abreu et al. (2011) found **2** in clone 23 and with subsequent assays of this and other SwAsp clones we discovered a second putative isomer, in addition to **2**, based upon LC–MS molecular weight data (Keefover-Ring et al., unpublished data). We kept the leaves in an ice chest while in the field and within 2 h returned them to the lab for immediate lyophilization and subsequent storage in sealed plastic bags at −20 °C. Prior to

Table 1
^{1}H and ^{13}C NMR data for compound **3** [2′-(*Z*)-cinnamoylsalicortin].

Position	^{1}H δ [ppm]	^{13}C δ [ppm]	COSY[a]	HMBC [ppm]
1′	5.01	99.0	2′	154.7
2′	5.07	73.7	1′, 3′	75.2, 99.0, 165.7
3′	3.66	75.2	2′, 4′	70.3, 73.7
4′	3.79	70.3	3′, 5′	75.2, 75.8
5′	3.46	75.8	4′, 6′	
6′a	3.83	61.8	5′	
6′b	3.91	61.8	5′	
1	–	154.7		
2	–	125.1		
3	7.25	129.8	4	63.6, 130.1, 154.7
4	7.06	123.2	3, 5, (6)	115.4, 125.1
5	7.30	130.1	6, 4	129.8, 154.7
6	6.97	115.4	(4), 5	123.2, 125.1, 154.7
7a	5.13	63.6		125.1, 129.8, 154.7, 169.5
7b	5.20	63.6		125.1, 129.8, 154.7, 169.5
8	–	169.5		
9	–	78.3		
10	5.77	127.5	11	26.7, 205.8
11	6.09	132.1	12, 10	26.7, 35.2, 78.3
12a	2.52	26.7	(10), 11, 13	
12b	2.68	26.7	(10), 11, 13	
13a	2.60	35.2	12	26.7, 132.1, 205.8
13b	2.98	35.2	12	26.7, 132.1, 205.8
14	–	205.8		
1″	–	165.7		
2″	6.01	118.7	3″	134.8, 145.0, 165.7
3″	7.06	145.0	2″	129.7
4″	–	134.8		
5″	7.54	129.7	6″	129.3, 145.0
6″	7.28	128.0	5″	
7″	7.28	129.3		

[a] Long range couplings shown in parenthesis.

extraction we ground all leaves (260 g DW) to a fine powder with a Tecator 1093 Cyclotec cyclone sample mill (Foss, Hillerød, Denmark), also storing the powder in sealed plastic bags at −20 °C, until needed.

3.2. Compound extraction and isolation

We used a modified version of the extraction procedure from Lindroth et al. (1986) to obtain a salicinoid-rich fraction. Briefly, we

Table 2
^{1}H and ^{13}C NMR data for compound **2** [2′-(*E*)-cinnamoylsalicortin].

Position	^{1}H δ [ppm]	^{13}C δ [ppm]	COSY	HMBC [ppm]
1′	5.16	99.4	2′	
2′	5.20	73.9	1′, 3′	75.3, 99.4, 166.8
3′	3.86	75.3	2′	70.3, 73.9
4′	3.88	70.3	5′	75.3
5′	3.55	75.9	4′, 6′	
6′a	3.92	61.7	5′	
6′b	3.94	61.7		
1	–	154.8		
2	–	125.2		
3	7.24	129.7	4′	63.7, 130.1, 154.8
4	7.05	123.3	3′, 5′	115.7, 125.2
5	7.30	130.1	6′, 4′	129.7, 154.8
6	7.08	115.7	5′	123.3, 125.2, 154.8
7a	5.15	63.7		125.2, 129.7, 154.8, 169.5
7b	5.18	63.7		125.2, 129.7, 154.8, 169.5
8	–	169.5		
9	–	78.3		
10	5.73	127.5	11	26.7, 205.9
11	6.06	132.0	10, 12	26.7, 35.2, 78.3
12a	2.49	26.7	11, 13	
12b	2.65	26.7	11, 13	
13a	2.58	35.2	12	78.3, 132.0, 205.9
13b	2.96	35.2	12	26.7, 132.0, 205.9
14	–	205.9		
1″	–	166.8		
2″	6.49	116.7	3″	134.1, 146.7, 166.8
3″	7.76	146.7	2″	116.7, 128.3, 134.1, 166.8
4″	–	134.1		
5″	7.52	128.3	6″, 7″	130.6
6″	7.37	128.9		
7″	7.37	130.6		

Table 3
UV maxima, theoretical and calculated exact masses, calculated molecular formulas, and main high-resolution tandem MS fragments of the two 2′-cinnamoylsalicortin isomers.

Compound	λ_{max}	m/z $(M-H)^-$			Tandem MS ion fragments (relative intensity)
		Theoretical	Calculated	Formula	
2′-(*E*)-Cinnamoylsalicortin (**2**)	218, 279	553.171	553.168	$C_{29}H_{29}O_{11}$	405 (100), 381 (16.0), 429 (12.9), 447 (10.1), 285 (8.4), 525 (7.2), 415 (5.3), 213 (7.0)
2′-(*Z*)-Cinnamoylsalicortin (**3**)	220, 273	553.171	553.169	$C_{29}H_{29}O_{11}$	405 (100), 429 (9.5), 381 (7.6), 553 (7.0), 447 (5.9), 415 (5.2), 525 (3.7), 285 (3.7)

initially extracted 215 g of lyophilized leaf powder with 1200 ml of a chilled (4 °C) mixture of MeOH–H_2O (80:20) for 1 h, vacuum filtered to remove the remaining plant material, and then partitioned the MeOH–H_2O with hexane–$CHCl_3$ (3:1, 400 ml ×2, 300 ml ×3), followed by further extraction of the MeOH–H_2O solution once with 700 ml $CHCl_3$. We then concentrated the remaining $CHCl_3$–MeOH solution to a brown crystalline solid, redissolved it in $CHCl_3$ followed by vacuum filtration and concentration, resulting in ~35 g of crude extract.

For compound separation with flash chromatography, we dissolved about 1 g of crude extract in 2 ml $CHCl_3$ and loaded it onto a 4 cm × 20 cm silica gel flash chromatography column. We eluted the column with $CHCl_3$–MeOH (16:1) and collected 20 ml fractions, which we monitored with TLC and/or LC–MS. We combined early eluting fractions which contained a mixture of **2** and the putative cinnamoylsalicortin isomer.

We further purified the flash fractions containing compounds **2** and **3** using a Gilson 333/334 preparative HPLC system with detection at 210 nm (Gilson 151) equipped with either a VP 150/10 (10 mm × 150 mm) or a VP 250/21 (21 mm × 250 mm) Nucleodur C_{18} HTEC 5 μm column (Macherey-Nagel GmbH & Co., Düren, Germany) with isocratic runs using H_2O:ACN (65:35; both with 0.005% formic acid) at flow rates of 7 and 20 ml min^{-1}, respectively. Purification runs concluded with consolidation of respective peaks from multiple injections, lyophilization, and dissolution in $CDCl_3$ for NMR analysis. Initial ^1H NMR results indicated that >20% of **3** isomerizes to **2** after lyophilization, possibly due to the aqueous acidic conditions; so for subsequent HPLC purifications of **3** we immediately extracted the pooled mobile phase collections twice with equal amounts of $CHCl_3$. The combined organic phases were then dried with anhydrous Na_2SO_4(s), filtered and concentrated which gave **3** as a colorless solid.

3.3. NMR analyses

We characterized the structures of **2** and **3** with ^1H and ^{13}C NMR on a Bruker DRX-400 spectrometer at 298 K with $CDCl_3$ as the solvent. We recorded one-dimensional ^1H and ^{13}C spectra and performed two-dimensional heteronuclear single-quantum correlation spectroscopy (HSQC), heteronuclear multiple-bond correlation spectroscopy (HMBC), correlation spectroscopy (COSY), and rotating-frame overhauser effect spectroscopy (ROESY) experiments. ^1H and ^{13}C chemical shifts were reported relative to $CHCl_3$ (δ_H 7.26 ppm) or $CDCl_3$ (δ_C 77.0 ppm) as internal references.

3.4. LC–MS and high-resolution tandem mass spectroscopy analyses

We initially used ultra-performance liquid chromatography with UV and time-of-flight mass spectrometer detectors (UPLC–UV-TOFMS) to measure the amounts of the two cinnamoylsalicortin isomers in the *P. tremula* leaves used for compound isolation, in flash fractions high in the two isomers and in the preparative HPLC collections of the individual purified compounds. For the lyophilized leaf powder analysis, we used a modified version of the sample extraction method and UPLC–UV-TOFMS conditions from Abreu et al. (2011) and quantified the two isomers using the compounds purified above as standards. For these analyses we used a Waters Acquity UPLC system equipped with an Acquity C_{18} column (2.1 mm × 100 mm, 1.7 μm, held at 40 °C) and a photodiode array detector coupled to an LCT Premier TOFMS in the negative ion mode (all from Waters, Milford, MA USA), using the same conditions as Abreu et al. (2011).

We gathered additional structural information for compounds **2** and **3** with high-resolution tandem mass spectrometry. We injected methanol solutions of both purified cinnamoylsalicortin isomers onto a Thermo Accela UPLC system with a Hypersil C_{18} GOLD column (2.1 mm × 50 mm, 1.9 μm, held at 40 °C). We then performed collision-induced dissociation (CID) at 35 eV with the deprotonated precursor ions (553 m/z for both isomers) with a LTQ/Orbitrap high-resolution mass spectrometer (all from Thermo Fisher Scientific, Bremen, Germany), also using the conditions of Abreu et al. (2011).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2013.11.012.

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