On the Metabolic Conversion of Salicortin-like Chemical Defenses (Salicortinoids) in the Lepidopteran Specialist Herbivore Cerura vinula (Notodontidae)

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Research Article

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Abstract

The lepidopteran specialist herbivore Cerura vinula (Notodontidae) has adapted to thrive on poplar and willow species (Salicaceae). Previous research showed that C. vinula uses a unique mechanism to detoxify the host plant's defense compounds. After discovering a reductively transformed derivative of tremulacin (6'-O-benzoyl-salicortinol) isolated from the frass of C. vinula, we assessed the chemical stability of salicortin-like defenses (salicortinoids) and analyzed their decomposition products over time and at different pH values. We then incubated uniformly $^{13}$C-labeled compounds in vitro with mid-gut homogenates of C. vinula larvae to determine the metabolism of salicortinoids. The key to the metabolic process, we found, is the initial reductive transformation of the salicortinoids; this step reduces the formation of toxic catechol.

Introduction

Plants of the genera Salix and Populus are chemically protected against herbivory by salicinoids. By definition, salicinoids contain saligenin substituted with a $\beta$-glucosyl moiety on its phenolic position. This glycosyl rest may contain another substitution in the 2' or the 6' position. In many salicinoids, the remaining benzylic position of saligenin is esterified with common acids such as benzoic acid, salicylic acid, or the structurally more complex 1-hydroxy-6-oxocyclohex-2-ene-1-carboxylic acid (6-HCH).(Boeckler et al. 2011) Salicinoids containing the 6-HCH moiety will hereafter be referred to as salicortinoids (Fig. 1A). How ingestion by the herbivore transforms the salicortinoids determines how the derived products act against the herbivore. Commonly, de-glucosylation occurs as salicortinoids pass through the insect gut, leading to the aglycons being excreted.(Lindroth 1988; Pentzold et al. 2014) Chemically reactive structures will be further transformed. In vitro studies showed that salicortinoids are not stable under acidic conditions, where they dehydrate and autoxidize to salicylate; under alkaline conditions, in contrast, ester cleavage, decarboxylation, and a subsequent transformation into catechol occurred. (Julkunen-Tiitto and Meier 1992; Pearl and Darling 1971; Ruuhola et al. 2003) Catechol and its oxidized form, ortho-quinone, were therefore considered as the principal toxin of plant-defense systems based on salicortinoids,(Appel 1993; Haruta et al. 2001) and high levels of salicortinoids were shown to reduce the growth of lepidopteran larvae (Fig. 1B).(Hemming and Lindroth 2000; Osier and Lindroth 2001; Ruuhola et al. 2001)

Recent in vivo studies with the generalist lepidopteran herbivore Lymantria dispar demonstrated that the metabolic breakdown of salicortinoids led to the accumulation of catechol, which was in turn metabolized to catechol glucoside, catechol glucoside phosphate, and N-acetylcystein catechol adducts. (Boeckler et al. 2016) However, studies with the lepidopteran specialist herbivore Cerura vinula showed that only quinic acid conjugates with salicylic acid and benzoic acid were produced by salicortinoid metabolism. Although a mechanism for this conversion was proposed, no metabolic intermediates corroborating the breakdown of salicortinoids were identified.(Feistel et al. 2018)
Here we report on the detailed degradation mechanism for reductively transformed salicortinoids, which, in our opinion, clarifies their conversion to salicylic acid. In our study, we used uniformly $^{13}$C-labeled compounds to avoid possible interference with metabolites sequestered by the insect.

**Methods And Materials**

**General Methods**

Nuclear magnetic resonance (NMR) spectra were recorded either on a Bruker Avance III HD 700 MHz spectrometer, equipped with a cryoplatform and a 1.7 mm TCI microcryoprobe, or on a Bruker Avance III HD 500 MHz NMR spectrometer, equipped with a cryoplatform and a 5 mm TCI cryoprobe (Bruker Biospin GmbH, Rheinstetten, Germany). All NMR spectra were recorded at 298 K with MeOH-d$_3$ as a solvent. Chemical shifts were referenced to the residual solvent peaks at $\delta_H$ 3.31 and $\delta_C$ 49.15. Data acquisition and processing were accomplished using Bruker TopSpin ver.3.6.1. Standard pulse programs as implemented in Bruker TopSpin ver.3.6.1. were used.

High-performance liquid chromatography coupled to high-resolution electrospray ionization mass spectrometry (HPLC-HR-ESI-MS) analyses were performed on an Agilent Infinity 1260 system, consisting of a combined degasser/quaternary pump G1311B, an autosampler G1367E, a column oven G1316A, and a photodiode array detector G1315D (Agilent Technologies GmbH, Waldbronn, Germany) connected to a Bruker Compact OTOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Standard parameters for small molecule analysis were used as implemented in Bruker Compass ver.1.9. Samples were measured in positive and negative ionization mode using a mass range of $m/z$ 100 to $m/z$ 700. An Agilent Poroshell 120 EC C-18 column, 2.7 µm, 4.6 x 50 mm, equipped with a Phenomenex SecurityGuard Cartridge C18, 4 x 3 mm (Phenomenex Ltd., Aschaffenburg, Germany), was used for separations. A binary solvent system of H$_2$O (solvent A) and acetonitrile (solvent B), both solvents containing 0.1% (v/v) formic acid, was used. The flow rate was set to 500 µl min$^{-1}$. The linear gradient started with 20% B and increased to 70% B within 13 min. The column was washed for 10 min with 100% B and re-equilibrated at 20% B for 5 min.

Preparative HPLC separations were accomplished using an Agilent 1100 HPLC system, consisting of a degasser G1322A, a binary pump G1312A, an autosampler G1313A, and a photodiode array detector G1315B. The column outlet was connected to an Advantec CHF122SB fraction collector (Advantec Toyo Kaisha Ltd., Tokyo, Japan) triggered by the HPLC via a relay contact board. All preparative HPLC separations were carried out using Macherey-Nagel (MN) columns (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Medium-pressure chromatographic (MPLC) separations were accomplished using a Biotage Isolera One chromatograph (Biotage Sweden AB, Uppsala, Sweden) using linear gradient elution on a 30 g Biotage Sfär C18 Duo column (solvents H$_2$O + 0.1% FA and MeOH + 0.1%FA). [U-$^{13}$C]salicortin, [U-$^{13}$C]HCH-salicortin, [U-$^{13}$C]tremulacin, and [U-$^{13}$C]trichocarpin were obtained from a methanolic extract of $^{13}$C-labeled material of *P. deltoides x trichocarpa* as described previously.[13] Homogenization of plant
material was carried out with a Bertin Minilys cell disruptor (Bertin Technologies, Montigny-le-Bretonneux, France). To purify \(^{13}\text{C}\)salicortin, \(^{13}\text{C}\)HCH-salicortin, and \(^{13}\text{C}\)tremulacin, a MN \(\pi^2\)-column (250 x 4.6 mm, 5 \(\mu\)m particle size) was used. To purify \(^{13}\text{C}\)trichocarpin, a MN phenyl-hexyl-column (250 x 4.6 mm, 5 \(\mu\)m particle size) was used. To purify 6-\(\text{O}\)-benzoyl-salicortin, a MN C18 HTec (250 x 10 mm, 5 \(\mu\)m particle size) was used. Detailed information on the purification procedures is given in the Supporting Information (SI). To recover the compounds from the fractions, solvents were evaporated using a Büchi rotary evaporator Rotavapor R-114 (Büchi Labortechnik, Flawil, Switzerland). Methanol (MeOH, LCMS grade) used for extraction and chromatographic separation was purchased from Merck KGaA (Darmstadt, Germany) and used without further purification. Water used for HPLC and HPLC-HR-ESI-MS separations was obtained from a Milli-Q Synthesis A10 purifier (Merck KGaA, Darmstadt, Germany). Acetonitrile (LCMS grade) and formic acid (eluent additive for LC-MS) used for HPLC-HR-ESI-MS analyses were purchased from Merck KGaA (Darmstadt, Germany). HR-X SPE cartridges (500 mg sorbent/6 mL volume and 200 mg sorbent/3 mL volume), folded paper filters (90 mm), and paper disc filters (MN 615 ¼, 125 mm) were purchased from Macherey-Nagel (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Syringe filters (0.45 \(\mu\)m, PA) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Salicin, catechol, saligenin, salicylic acid, and benzoic acid were purchased from Merck KGaA (Darmstadt, Germany). For centrifugations, an Eppendorf Centrifuge 5415 R (Eppendorf SE, Hamburg, Germany) was used.

**Plant Material**

Hybrid trembling aspen (\(\text{Populus tremula x tremuloides}\)) and \(\text{P. deltoides x trichocarpa}\) were grown outdoors at the greenhouse facilities of the Max Planck Institute for Chemical Ecology in Jena, Germany. Puss moth larvae (\(\text{C. vinula}\)), which are reared at the outdoor butterfly facility of the Max Planck Institute for Chemical Ecology in Jena, Germany, were used for metabolic studies.

**Extraction of \(\text{C. vinula}\) frass and isolation of 6’-\(\text{O}\)-benzoyl-salicortinol**

Frass (20 g, dry weight) of \(\text{C. vinula}\) fed on \(\text{P. tremula x tremuloides}\) was crushed in a mortar and extracted with MeOH (5 x 100 mL) in an Erlenmeyer flask. After filtration through a folded paper filter the combined extracts were filtered through a cartridge filled with HR-X sorbent (30 mL) to remove small particles and very lipophilic compounds, e.g. fatty acids and chlorophyll. The filtrate was rotary-evaporated to yield the crude extract (2.92 g). This crude extract was reconstituted with MeOH, and 800 mg of HR-X sorbent was added to adsorb the extract completely. To separate the crude extract coarsely, a cartridge (60 mL) filled with HR-X resin (5.0 g) was equilibrated with MeOH (100 mL) and conditioned with \(\text{H}_2\text{O}\) (100 mL). Afterwards, the HR-X resin loaded with the crude extract was applied on top of the cartridge, and a sintered PP filter disc was inserted to compress the bed. A stepwise gradient elution was applied using a binary solvent system (\(\text{H}_2\text{O}\) (A)/ MeOH (B), 0% φ 100% B in 10% steps). For each step, a volume of 100 mL of the respective solvent mixture was used, and fractions of 50 mL were collected. In total, 23 fractions were obtained, and an aliquot of each was subjected to HPLC-HR-ESI-MS analysis. All
fractions were then evaporated to dryness, and their weight was determined (see SI, Table 1). Based on results of the analysis, the fraction 90%-I was subjected to separation by MPLC (Biotage Isolera One). The MPLC gradient started with 0% (two cartridge volumes, CVs) of MeOH (solvent B) and increased during the elution of one CV to 10% B. Afterwards, B increased within 42 CVs to 70% The column was purged with seven CVs of 100% B. Aliquots of the MPLC fractions were subjected to HPLC-HR-ESI-MS analysis. Dry weights of the MPLC fractions are listed in SI, Table 2. MPLC fraction seven (F7), containing 6'-O-benzoyl-salicortinol, was reconstituted with 300 µL MeOH (47.5 mg mL$^{-1}$) and separated by semi-preparative HPLC (32% MeOH in H$_2$O, 70 min isocratic elution at 3.5 mL min$^{-1}$ flow). After each run, the column was purged for 10 min with 100% MeOH and equilibrated for 10 min at initial conditions. The fraction containing 6'-O-benzoyl-salicortinol (rt 27.2 min) was evaporated using N$_2$ gas. The structure of the isolated compound was confirmed by HPLC-HR-ESI-MS and NMR spectroscopy (see SI, Fig. 1–8 and Table 3). The peak for 6'-O-benzoyl-salicortinol appeared at R$_t$ = 11.6 min in HPLC-HR-ESI-MS.

**Isolation of [U-$^{13}$C]salicortin, [U-$^{13}$C]HCH-salicortin and [U-$^{13}$C]tremulacin**

An extract of $^{13}$C-labeled *P. deltoides x trichocarpa* leaf material, prepared as described previously, was obtained [13], and 2.19 g dried extract was used to isolate compounds. After being reconstituted with MeOH, aliquots (146.19 mg ml$^{-1}$) were subjected to HPLC separation using chromatographic conditions as described previously.(Feistel et al. 2018) Subsequently, the isolated compounds were purified for a second time to remove traces of impurities. The obtained yields were as follows: 48.2 mg of [U-$^{13}$C]salicortin, 12.9 mg of [U-$^{13}$C]trichocarpin, 45.1 mg of [U-$^{13}$C]HCH-salicortin, and 21.5 mg of [U-$^{13}$C]tremulacin. For details of the re-purification and analytical data of the purified $^{13}$C-labeled compounds, see SI. The calculation of $^{13}$C-enrichment of the $^{13}$C-labeled plant metabolites was accomplished as described in Taubert et al.(Taubert et al. 2011) The in vivo-generated [U-$^{13}$C]salicortin showed 82% total $^{13}$C-enrichment, [U-$^{13}$C]HCH-salicortin showed 77% total $^{13}$C-enrichment, and [U-$^{13}$C]tremulacin showed 79% total $^{13}$C-enrichment. Spectroscopic data of these compounds and detailed information about the $^{13}$C-enrichment are shown in SI (Fig. 9 - Fig. 36, Table 4 - Table 9).

**Decomposition of 6'-O-benzoyl-salicortinol at pH 6 to pH 9**

To determine the decomposition products of 6'-O-benzoyl-salicortinol at various pH values, a stock solution of 2 mg mL$^{-1}$ in acetonitril was prepared. Phosphate-buffered saline (PBS, 150 µl) adjusted to pH 6, pH 7, pH 8, and pH 9, respectively, was pipetted into a HPLC vial equipped with a 200 µl insert; 7.5 µL of the 6'-O-benzoyl-salicortinol stock solution was mixed in using several pipette strokes. The experiments were carried out at room temperature (25°C). Decomposition of the compounds was measured every 2 h by HPLC-HR-ESI-MS using the same method as described above (for results see also, SI Fig. 37).

**Spontaneous degradation of salicortinoids at pH 7.8**
To examine the spontaneous decomposition of salicortinoids, a stock solution of the respective compound (at a concentration of 1 mg ml\(^{-1}\) in MeOH) was prepared. An aliquot of 10 µL of this stock solution was diluted with 90 µL of PBS (pH 7.8). The pH value was chosen on the basis of previous publications and results from our own experiments to determine the mid-gut pH of \(C. vinula\) larvae. (Feistel 2018) Decomposition of the salicortinoids was monitored every 30 min by means of HPLC-HR-ESI-MS analysis as described above (SI Fig. 43). As a control, the first analysis was done right after the salicortinoid aliquot was mixed with PBS.

**Sampling of larvae and their dissection**

\(C. vinula\) larvae (5th instar) raised on \(P. deltoides \times trichocarpa\) leaves were immobilized in a Falcon tube and kept at -20°C for 15 min. A midabdominal leg was removed with scissors, and the emerging hemolymph was absorbed with a tissue. Afterwards the caterpillar was opened by a ventral cut. The mid-gut was separated from the fore- and hind-guts, and malphigian tubulae were removed from the gut tissue with tweezers. The mid-gut was emptied and rinsed with PBS, pH 7.8. During the dissection procedure, samples were stored on ice and kept at -80°C until further use.

**Incubation of gut homogenates with \[^{13}\text{C}\] labelled salicortinoids**

Mid-gut tissue kept at -80°C was thawed on ice and homogenized in a tissue grinder with 1 mL chilled PBS (pH 7.8). The gut homogenate was divided into two 500 µL portions and transferred into 1.5 mL Eppendorf tubes. For the assay, 500 µL gut homogenate, 500 µL PBS (pH 7.8), and 10 µL salicortinoid stock solution in dimethylsulfoxide (DMSO, concentration 10 mg/mL) were mixed. For background determination, 500 µL gut homogenate, 500 µL PBS (pH 7.8), and 10 µL DMSO was used. The incubation was carried out at room temperature (25°C). For sampling, aliquots (100 µL) of both groups were taken initially after mixing and at 0.5 h, 1 h, 1.5 h, 2h, 3h, and 4 h. Prior to the analyses, the decomposition reaction was quenched by the addition of 0.1 % formic acid in methanol (100 µL). Samples were then centrifuged at 4°C for 10 min at 13200 rpm/16100 rcf. The supernatant was transferred into a HPLC vial and subjected to HPLC-HR-ESI-MS analysis using the same conditions as described above.

**Results**

**The frass constituent 6’-O-benzoyl-salicortinol results from reductively transformed tremulacin**

We identified a compound with the molecular formula \(\text{C}_{27}\text{H}_{30}\text{O}_{11}\) in the frass of \(C. vinula\) larvae raised on \(P. deltoides \times trichocarpa\) leaves (\(m/z\) 529.1728 [M-H]\(^{-}\), calc. for \(\text{C}_{27}\text{H}_{29}\text{O}_{11}\)\(^{-}\), \(m/z\) 529.1715). The main fragment ion of the compound was determined at \(m/z\) 389.1267 [M-H]\(^{-}\), corresponding to a molecular formula of \(\text{C}_{20}\text{H}_{22}\text{O}_{8}\). The molecular composition suggested the presence of tremuloidin or populin. Another main fragment of this compound displayed a HRESIMS ion at \(m/z\) 157.0508 [M-H]\(^{-}\), indicating a
sum formula of $\text{C}_7\text{H}_9\text{O}_4^-$ (calc. for $\text{C}_7\text{H}_9\text{O}_4^-$, $m/z$ 157.0506). Analysis of the fragmentation pattern suggested a reductively transformed tremulacin-like compound. Using NMR spectroscopy, we identified the structure as 6'-O-benzoyl-salicortinol. The 6-HCH moiety was reduced to 1,6-dihydroxycyclohex-2-ene-1-carboxylate (hereafter referred to as DHCH). Comparison with the literature led us to define the stereochemistry as (S)-configured at C1' and (R)-configured at C6' (SI Fig. 1–8 and SI Table 3). (Wei et al. 2015)

**The in vitro decomposition of 6'-O-benzoyl-salicortinol is pH dependent**

To investigate the chemical stability of 6'-O-benzoyl-salicortinol, an equimolar amount of the compound was dissolved in PBS adjusted to pH 6, pH 7, pH 8, and pH 9, and its decomposition products were analyzed by HPLC-HR-ESI-MS. Samples were taken every 2 h over a time course of 22 h. Although only 2% (pH 6) – 18% (pH 9) of the compound decomposed, we identified the same decomposition products for all tested pH values: salicortinol, populin, salicin, DHCH, and salicylic acid. Remarkably, no catechol formation was observed. The highest amounts of salicortinol, populin, salicin, and DHCH were observed at pH 9. The decomposition at pH 8 was substantially slower than at pH 9, and the slowest decomposition was observed at pH 6. The highest amount of salicylic acid was formed at pH 6. The second highest amount of salicylic acid was formed at pH 9, and the lowest was produced at pH 7. Based on these observations, we assume that salicylic acid is formed under either alkaline or acidic conditions, while noting that acidic conditions catalyzed the 6-DHCH conversion to salicylic acid most efficiently (SI Fig. 37–42).

**Salicortinoids degrade slowly in vitro at pH 7.8**

To determine the chemical stability of salicortinoids at conditions present in the gut of *C. vinula* larvae, *in vitro* hydrolysis experiments were conducted. 100 µg of salicortinoid was incubated at 25°C, and decomposition was monitored every 30 min by HPLC-HR-ESI-MS analysis. Spontaneous salicortinoid decomposition was slow at pH 7.8. After 7.5 h of incubation, 73% of salicortin, 45% of HCH-salicortin, and 69% tremulacin remained. Hydrolysis products of salicortin generated *in vitro* were determined to be salicin and catechol. For HCH-salicortin, the hydrolysis products were salicortin, salicin, and catechol. In comparison to salicortin, HCH-salicortin generated about twice as much catechol as was found in the peak areas of the HPLC-HR-ESI-MS chromatogram (see SI Fig. 48). This increase indicated that the HCH-moiety spontaneously degraded to catechol under slightly alkaline conditions. Salicortin, salicin, catechol, and also tremuloidin and populin were identified as degradation products of tremulacin, suggesting that an ester cleavage liberated the HCH-moiety, which was then oxidized to catechol (Fig. 2, SI Fig. 43 - Fig. 47).

**Salicortinoids degrade quickly in mid-gut homogenate at pH 7.8**
From our in vitro incubation experiments with gut homogenates at pH 7.8, we concluded that all tested salicortinoids were completely degraded within four hours and more than 50 percent were already degraded within half an hour. Even during the initial mixing of the salicortinoids with the gut homogenate, a reduction of the 6-HCH-moiety was observed. Salicortinol and tremulacinol degrade at the same rate as salicortin and tremulacin, respectively. For all salicortinoids, we observed $^{13}$C-labeled salicin, saligenin, salicylic acid, and DHCH. The $^{13}$C-labeling grade of these metabolites was determined to correspond to the $^{13}$C-labelling grade of the salicortinoids used. For tremulacin, we detected benzyolated compounds such as tremuloidin and populin. Catechol was also generated in all incubation experiments but in lower concentrations compared to those determined for the in vitro degradation experiments (SI Fig. 49 - Fig. 66).

**Discussion**

Earlier in vitro studies of the metabolism of salicortinoids assumed that degradation by β-glucosidases and esterases leads to the release of saligenin and the 6-HCH moiety.(Julkunen-Tiitto and Meier 1992; Lindroth 1988) The latter is further oxidized to catechol and/or ortho-quinone under the alkaline conditions present in lepidopteran mid-guts.(Appel 1993; Appel and Martin 1990; Harrison 2001) The ortho-quinone is believed to cause harm to herbivores by protein cross-linking.(Felton et al. 1992; Haruta et al. 2001) Recent research on C. vinula established an alternative pathway for the metabolism of salicortinoids based on observations that larvae fed on [U-$^{13}$C]salicortin formed only salicylic acid conjugates. This observation led to the conclusion that both parts of salicortin, saligenin, and the 6-HCH moiety were transformed to salicylic acid.(Feistel et al. 2018) However, the mechanism of this transformation remained unclear.

For the present study, we raised C. vinula larvae on P. tremula x tremuloides, a species whose leaves contain high amounts of the salicortinoids tremulacin and salicortin (see SI Fig. 67). In addition to the frass metabolites described by Feistel et al.(Feistel et al. 2018), we isolated another compound, 6′-O-benzoyl-salicortinol. Because of its structural similarity, we assumed this compound was produced by the metabolic transformation of tremulacin. Acylated salicinoids are known to resist chemical and enzymatic degradation.(Julkunen-Tiitto and Meier 1992; Ruuhola et al. 2003) The insect in the present study was ingesting salicortin and tremulacin, but only the tremulacin metabolite remained, suggesting that the reduced form of salicortin, salicortinol, was very quickly degraded and therefore not isolable. Also, there is evidence that acyl-substituents in glucosyl moieties tend to migrate to position 6′, where the most stable derivative is formed.(Pearl and Darling 1963; Romanova et al. 2020) We therefore deduced 6′-O-benzoyl-salicortinol to be an early rearranged intermediate of the tremulacin metabolism and hypothesize that its benzyolation hinders degradation by the enzymes that otherwise quickly break down salicortin. It has often been shown that β-keto acids decarboxylate during metabolic transformation,(Pollack 1978), but in the present case, the ketone of 6-HCH is reduced to an alcohol. This reduction leads to DHCH formation under physiological conditions that subsequently prevent the formation of catechol (Fig. 3). The isolated 6′-O-benzoyl-salicortinol was incubated at 25°C in PBS at pH 6, pH 7, pH 8, and pH 9 to test its stability.
and to identify possible decomposition products. The compound proved to be stable, and only 2% (pH 6) – 18% (pH 9) decomposed during the experimental time of 22 h. We could, however, determine the structures of the breakdown products (see SI Fig. 37 – Fig. 42). The decomposition products, results of ester hydrolyses, were elucidated as populin, salicortinol, salicin, and DHCH. We also identified salicylic acid as a breakdown product. (Fig. 3). (Ruuhola et al. 2003) Ester cleavage, which is catalyzed by alkaline conditions, illustrates the trend that the higher the pH values, the faster the degradation.

It was earlier suggested that the pH-dependent degradation of salicortinoids starts with the release of the 6-HCH moiety. (Ruuhola et al. 2003) Accordingly, we proposed that ester cleavage occurs at the benzylic position as the first step in the decomposition of 6'-O-benzoyl-salicortinol, leading to populin and DHCH. In contrast to former studies, (Julkunen-Tiitto and Meier 1992; Ruuhola et al. 2003) benzoic acid was observed to cleave from the glucose moiety liberating salicortinol (Fig. 3). In addition, we observed the de-benzylation of tremulacin in our salicortinoid-decomposition experiment. Similarly, the de-benzylation of populin lead to salicin. Another source of salicin is the hydrolysis of the benzylic ester of salicortinol, producing free DHCH, which can then form salicylic acid under acidic or alkaline conditions. This transformation happens most likely by dehydration and subsequent auto-oxidation (see SI Fig. 69).

To compare the stability of 6'-O-benzoyl-salicortinol with that of other salicortinoids, we tested salicortin, HCH-salicortin, and tremulacin in vitro at pH-conditions present in the mid-gut of C. vinula (pH 7.8). The salicortinoids quickly decomposed under strong alkaline conditions, but at pH 7.8, most of the three tested salicortinoids remained intact until the end of the experiment (for 7.5 h). Salicortin hydrolyzed to salicin and catechol, HCH-salicortin to salicortin, salicin, and catechol. Unsurprisingly, HCH-salicortin released twice as much catechol as salicortin (the HCH-moiety in salicortinoids has been identified as the source of catechol under alkaline conditions).[4–6] Decomposition products of tremulacin were catechol, salicortin, salicin, tremuloidin, and populin. In tremuloidin, we observed acyl-migration from the 2'-OH group to the 6'-OH group of the glucosyl part. (Pearl and Darling 1963) In summary, we found the physiological pH of 7.8 hindered the spontaneous degradation of salicortinoids.

Several studies have utilized mid-gut homogenate or enzyme preparations to elucidate the metabolism of plant xenobiotics. (Lindroth 1988; Marty and Krieger 1984; Wouters et al. 2014) In our study, when samples were incubated with mid-gut homogenate at pH 7.8, the tested salicortinoids were completely degraded within 4 hours. We therefore concluded that degradation was enzymatic because our in vitro experiments with the buffer solutions left all salicortinoids intact. For all three salicortinoids, we identified the breakdown products salicortinol, salicin, saligenin, DHCH, salicylic acid, and catechol. Additionally, tremulacinol was formed from tremulacin.

Our results indicate that reductive transformation is the first step of salicortinoid metabolism in this specialist herbivore. Reductive metabolic transformation was also shown to be the key event in the detoxification of the cardenolide uscharidin to calactin and calotropin in Danaus plexipus and in the breakdown of juglone to 1,4,5-trihydroxynaphthalene in Cydia pomonella and Grapholita (= Cydia) molesta. (Marty and Krieger 1984; Piskorski and Dorn 2011; Piskorski et al. 2011) Reducing the keto-6-
HCH moiety to the corresponding alcohol prevents larger amounts of the toxin catechol from forming. Only 13% of salicortin, 14% of HCH-salicortin and 19% of tremulacin were transformed to catechol by the prevailing pathway (Fig. 1B), while the major part of salicortinoids were reduced. After the reduced salicortinoids are formed, the ester bond is hydrolyzed to yield salicin and free DHCH. Salicin is then deglucosylated to form saligenin. These previously reported transformations are regarded as essential steps in salicinoid metabolism. (Boeckler et al. 2016; Feistel et al. 2018; Julkunen-Tiitto and Meier 1992; Ruuhola et al. 2003) In vivo studies with Lymantria dispar described no further metabolism of saligenin, whereas studies with C. vinula pointed to its oxidative metabolism. (Boeckler et al. 2016; Feistel et al. 2018) Also our results indicate that saligenin is further oxidized to salicylic acid. We assume that such oxidation is facilitated by enzymes of the cytochrome P450 family. The involvement of cytochrome P450 in the transformation of xenobiotics or plant defensive compounds is well established in the study of plant-herbivore interaction. (Calla et al. 2017; Li et al. 2003; Schuler 2012) Another source of salicylic acid is DHCH, which can be converted to salicylic acid under alkaline mid-gut conditions (Fig. 4), and oxidizing enzymes from the cytochrome P450 family might accelerate its transformation. We did not observe the occurrence of the free salicortinol aglycon. Most likely this compound is less stable compared to its glucosylated counterpart and decomposes quickly to saligenin and DHCH. Such instability was also observed for the aglycon of salicortin. (Julkunen-Tiitto and Meier 1992; Lindroth 1988)

The metabolism of HCH-salicortin is similar to that of salicortin (Fig. 4A). Although we did not find reduced forms of HCH-salicortin, we did observe the formation of salicortinol. Both HCH-moieties of HCH-salicortin seemed to be reduced, since the signal intensity of free DHCH is highest for HCH-salicortin. The additional HCH-moiety is initially cleaved off from the glucosylated form, resulting in salicortin, which then undergoes the breakdown as described above.

The metabolism of tremulacin (Fig. 4B) is similar to that of salicortin. In the initial step, tremulacin is reduced to tremulacinol. After tremulacinol was hydrolyzed, we observed salicortinol, as well as tremuloidin, populin, and 6-DHCH. Salicortinol and DHCH were metabolized as described above. We observed the migration of the benzoyl moiety of tremuloidin to position 6'-OH and also an ester cleavage. The resulting salicin was then transformed as described for salicortin. In previous in vitro experiments, where only single enzymes from non-insect sources were used, the glycosidic esters were reported to remain intact. (Julkunen-Tiitto and Meier 1992; Ruuhola et al. 2003) In the experiments described here we used C. vinula mid-gut homogenates, which contain a variety of salicortinoid-degrading enzymes. This may explain why our results resemble those of in vivo experiments with Ophera brumata and L. dispar. In both species, glycosidic esters were hydrolyzed. (Boeckler et al. 2016; Ruuhola et al. 2001)

The formation of catechol was observed for all tested salicortinoids when samples were incubated with the gut homogenate. Interestingly, the compound was only formed initially, but in the course of the experiment its concentration decreased, probably due to protein binding. (Felton et al. 1992) The initially formed catechol is attributable to the uncontrolled action of glucosidases and esterases, which was described in other in vitro studies. (Julkunen-Tiitto and Meier 1992) Notably, during the gut homogenate incubation, the concentration of catechol never reached that observed in the buffer-only decomposition
experiments, although more than 50% of the salicortinoids were degraded within the first 30 min. Instead of being oxidized, 6-HCH was reduced, as evident from the high amount of free DHCH. Whether an insect will benefit from the suppression of all catechol is questionable. Previous studies have shown that the oxidized form of catechol, ortho-quinone, can bind to the occlusion bodies of the nuclear polyhedrosis virus. (Felton and Duffey 1990) This binding reduces the infectivity of the virus and improves larval survival rates after a viral challenge. (Ali et al. 1999; Wan et al. 2018; Wang et al. 2020)

In this study, we identified the metabolic key transformation that initiates the detoxification of salicortinoids in larvae of the lepidopteran specialist herbivore C. vinula. Tremulacin, an abundant salicortinoid in members of the Salicaceae family, was transformed into 6'-O-benzoyl-salicortinol, which was recovered from the insect's frass. The latter compound could be further broken down enzymatically into populin, salicortinol, free DHCH, and salicylic acid when incubated with mid-gut homogenate at pH 7.8. Catechol, regarded as the main toxic salicortinoid transformation product, was produced only from 13% of salicortin, 14% of HCH-salicortin and 19% of tremulacin. However, when salicortinoids were incubated in buffers of pH7.8, spontaneous degradation raised concentrations of catechol. We therefore propose that to avoid producing catechol, larvae engage in an initial reductive transformation of salicortinoids. Proof for this hypothesis came from incubation experiments with C. vinula mid-gut homogenate for salicortin, HCH-salicortin, and tremulacin. Our studies revealed a previously unknown deactivation mechanism for salicortinoids. To the best of our knowledge, this metabolic phase I reduction occurs only rarely in processes to detoxify plant chemical defenses. We believe that a similar mechanism for deactivating salicortinoids also evolved in closely related species of the Notodontidae family.

Declarations

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Contributions

Experimental design, insect rearing, data collection, and data analysis: FS. Structure elucidation: CP. The manuscript was drafted by FS and CP.

Ethics declarations

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Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

References


Figures

A

salicortinoids & salicortinols from Salix & Populus

salicortin (1): $R^1 = H, R^2 = H$
HCH-salicortin (2): $R^1 = H, R^2 = 6$-HCH
tremulacin (3): $R^1 =$ benzoyl $R^2 = H$

salicortinol (4): $R^1 = H, R^2 = H$
tremulacinol (5): $R^1 =$ benzoyl $R^2 = H$

6-O-benzoyl-salicortinol (6): $R^1 = H, R^2 =$ benzoyl

B

Mechanism of chemical defense by salicortinoids deduced from in vitro studies

enzymes

saligenin (7) + 6-hydroxycyclohexenonate (6-HCH) (8) → ortho-quinone (9)

Figure 1

Naturally occurring salicortinoids and salicortinols (A), Proposed mechanism for how salicortinoids break down into toxic products (B)
Figure 2

Decomposition of salicortinoids in PBS at pH 7.8

salicortin (1): $R_1 = \text{-}H, R_2 = \text{-}H$

HCH-salicortin (2): $R_1 = \text{-}H, R_2 = \text{-}HCH$

tremulacin (3): $R_1 = \text{-}\text{benzoyl}, R_2 = \text{H}$

salicin (10): $R_1 = \text{-}H, R_2 = \text{-}H$

tremuloidin (11): $R_1 = \text{-}\text{benzoyl}, R_2 = \text{H}$

populin (12): $R_1 = \text{-}H, R_2 = \text{-}\text{benzoyl}$

catechol (13)

Figure 3

Decomposition of 6'-O-benzoyl-salicortinol in PBS at pH 6 to pH 9

BA = benzoic acid (16)

DHCH (14)

salicylic acid (15)
Figure 4

Transformation of the salicortinoids salicortin and HCH-salicortin (A) and tremulacin (B) by C. vinula mid-gut homogenates

Supplementary Files

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