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# A Novel Zwitterionic Anthocyanin from Evergreen Blackberry (*Rubus laciniatus* Willd.)

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A novel zwitterionic anthocyanin was isolated from evergreen blackberry (*Rubus laciniatus* Willd.) and structurally characterized as cyanidin 3-dioxalylglucoside. During short-term storage in acidified methanol, methyl esterification of the carboxyl group of the terminal oxalyl unit occurred, providing additional information for structural elucidation. Until now, oxalic acid as an acyl moiety of anthocyanins has been reported only in orchid flowers (Orchidaceae) but never in fruits or in the Rosaceae. This is the first report of an anthocyanin diacylated with oxalic acid, establishing a more widespread occurrence of anthocyanins substituted with aliphatic acids than hitherto believed.

**KEYWORDS: Evergreen blackberry; *Rubus laciniatus* Willd.; acylated anthocyanin; aliphatic acid; oxalic acid; zwitterionic anthocyanin**

## INTRODUCTION

It is only in recent years that anthocyanins acylated with aliphatic acids have been characterized (*1*−*4*). Because of their lability to mineral acids during extraction, their presence has been previously overlooked (*5*). They display the same spectral properties as nonacylated anthocyanins, so they were often thought to be glycosylated structures (*6*). In addition to acetic, butyric, 2-methylbutyric, and tiglic acids, the following dicarboxylic structures have been reported as aliphatic acyl moieties in anthocyanins: malonic, malic, oxalic, and succinic acids (*5*, *7*).

Cyanidin 3-glucoside and cyanidin 3-rutinoside have long been established as the respective major and minor anthocyanins in blackberries (*8*). Sapers et al. (*9*) showed in addition the presence of cyanidin 3-xyloside along with two cyanidin derivatives, which were vaguely characterized as being substituted with dicarboxylic acids. Rommel et al. (*10*) showed that the acylated pigments had greater stability during fermentation and were also resistant to degradation by commercial pectinase enzyme preparations. Fan-Chiang (*11*) identified one of these pigments as cyanidin 3-glucoside acylated with malonic acid using electrospray mass spectrometry along with HPLC as well as hydrolysis and saponification reactions. Sapers et al. (*9*) also reported that the least polar of the two acylated pigments was the main pigment (50.5%) in unripe blackberries of the cultivar Hull Thornless (*Rubus* sp. L.), decreasing dramatically during the ripening process. Our objective was to further characterize this pigment with its interesting biological and chemical properties.

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## MATERIALS AND METHODS

**Pigment Material.** Fresh ripe fruit of *Rubus laciniatus* Willd., evergreen blackberry, was provided by International Flavor and Fragrances Inc. (Woodburn, OR). Evergreen blackberry juice concentrate was supplied by IFF Flavors North America (Salem, OR).

**Pigment Extraction.** Frozen evergreen blackberries (40 g) were liquid nitrogen powdered using a stainless steel Waring Blendor as previously described (*12*). The powder was then extracted with acetone (1:1, w/v) and centrifuged (3000 rpm, 20 min). The sediment was treated with aqueous acetone (30:70, v/v) until it was visually determined that no further anthocyanin could be extracted. Subsequently, the supernatants were combined and mixed with chloroform (1:2, v/v). After centrifugation (3000 rpm, 20 min), the aqueous phase was collected, evaporated in vacuo at 35 °C until all residual acetone was removed, and then made up to a known volume with purified water. When commercial blackberry juice concentrate was used as the pigment source, it was first diluted 1:5 (w/w) with purified water.

**Anthocyanin Semipurification.** For semipurification with $C_{18}$ Sep-Pak cartridges (Waters Associates, Milford, MA) the method of Wrolstad et al. (*13*) was slightly modified. After the sorbent had been activated with methanol, it was preconditioned with 1% aqueous formic acid and the anthocyanin-containing samples were applied to the minicolumn. Sugars, acids, and other water-soluble compounds were removed with 3 volumes of 1% aqueous formic acid. Rinsing with 2 volumes of ethyl acetate eluted phenolics as previously described (*14*). Anthocyanins were subsequently recovered by methanol containing concentrated formic acid (95:5, v/v) and evaporated in vacuo at 35 °C. Pigment solutions were diluted with purified water containing 1% aqueous formic acid and used for semipreparative HPLC.

**Pigment Isolation.** The unknown anthocyanin was isolated from the semipurified sample by semipreparative HPLC (system III, see below). The collected fraction was passed through a $C_{18}$ Sep-Pak cartridge as described above, except that the isolated anthocyanin was recovered by elution with 100% methanol. The methanol was reduced at 35 °C using a rotary evaporator, and pure methanol was repeatedly

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added to facilitate removal of acid and water remaining in the sample. The flask containing the dried anthocyanin was stored overnight in a desiccator. The pigment was then transferred to a screw-top test tube, flushed with nitrogen, and stored at −70 °C. The estimated overall yield was 1.5 mg.

Purity of the isolated pigment was checked by HPLC using system I (see below), monitoring the spectra of the peaks at 280, 320, and 520 nm. The peak area of the isolated pigment compared to the whole chromatogram area at 280 nm was used to evaluate purity and was found to be >95%.

**Acid Hydrolysis of Anthocyanins.** Acid hydrolysis was performed following the procedure described previously (*15*). Ten milliliters of 2 N HCl was added to the semipurified sample, and the solution was then hydrolyzed for 30 min under nitrogen atmosphere at 100 °C. After cooling in an ice bath, the colored solution was applied to an activated $C_{18}$ Sep-Pak cartridge and the latter rinsed with 2 volumes of purified water. Before the anthocyanin fraction was eluted with acidified methanol, 2 volumes of ethyl acetate was passed through the cartridge to remove uncolored compounds. The methanol fraction was reduced in vacuo at 35 °C, redissolved in 4% phosphoric acid, and submitted to HPLC system II.

**Alkaline Hydrolysis of Anthocyanins and Organic Acid Analysis.** As previously described (*16*), the purified pigment was saponified in a screw-top test tube by the addition of 1 mL of 10% aqueous KOH and kept for 8 min at room temperature in the dark. Then 2 N HCl was slowly added under ice cooling, until the solution turned red again. Using a $C_{18}$ Sep-Pak cartridge, the saponified anthocyanin was subsequently semipurified as described above and analyzed by HPLC using system I. The fraction eluting with purified water, which contained the acid substituent, was collected and acidified to pH 2 by the addition of dilute HCl before it was partitioned three times with ethyl acetate. The combined organic phases were reduced in vacuo at 35 °C and redissolved in purified water. Organic acid analysis was performed using system IV, and retention times were compared to those of organic acid standard solutions.

**High-Performance Liquid Chromatography (HPLC).** *(A) Analytical HPLC for Anthocyanins (System I) and Anthocyanidins (System II). (1) Apparatus.* A Perkin-Elmer series 400 (Norwalk, CT) high-performance liquid chromatograph was used, equipped with a 50 μL injection loop and a Hewlett-Packard 1040A photodiode array detector (Waldbronn, Germany). Data were analyzed with Hewlett-Packard HPLC$^{2D}$ ChemStation software (Waldbronn, Germany).

*(2) Columns, Mobile Phases, and HPLC Conditions.* Chromatographic analysis was done according to the method of Durst and Wrolstad (*15*) using an analytical scale (250 mm × 4.6 mm i.d.) Prodigy $C_{18}$ reversed phase column with a particle size of 5 μm (Phenomenex, Torrance, CA), fitted with a security guard $C_{18}$ ODS (4 mm × 3.0 mm i.d.). Simultaneous monitoring was performed at 280, 320, and 520 nm at a flow rate of 1 mL/min. Mobile phase A consisted of 100% HPLC grade acetonitrile, and mobile phase B was a mixture of 1% phosphoric acid (85%), 10% acetic acid (glacial), and 5% acetonitrile (v/v/v) in water. Solvents and samples were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA).

*(3) System I.* Separation of anthocyanins was achieved within 25 min. The first 5 min was performed isocratically with 100% B, followed by a linear gradient from 100% B to 80% B in 15 min and then to 60% B in 5 min.

*(4) System II.* Separation of anthocyanidins was performed by following a linear gradient from 95% B to 80% B in 20 min.

*(B) Semipreparative HPLC of Anthocyanins (System III). (1) Apparatus.* A semipreparative Dynamax Rainin model SD-300 high-performance liquid chromatograph (Woburn, MA) was used, equipped with a 1 mL injection loop and a Hewlett-Packard 1040A photodiode array detector. Data were analyzed with Hewlett-Packard HPLC$^{2D}$ ChemStation software.

*(2) Columns, Mobile Phases, and HPLC Conditions.* Semipreparative HPLC was performed using a semipreparative scale (250 mm × 21.4 mm i.d.) Microsorb $C_{18}$ reversed phase column with a particle size of 5 μm (Rainin Instrument Co., Inc., Emeryville, CA), fitted with a guard module $C_{18}$ ODS (50 mm × 21.4 mm i.d.). Monitoring was at 520 nm at a flow rate of 12 mL/min. Mobile phase A consisted of 100% HPLC grade acetonitrile, and mobile phase B was a mixture of 1% phosphoric acid (85%), 10% acetic acid (glacial), and 5% acetonitrile (v/v/v) in water. A linear gradient was followed from 92% B to 82% B in 15 min. Solvents and samples were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp.).

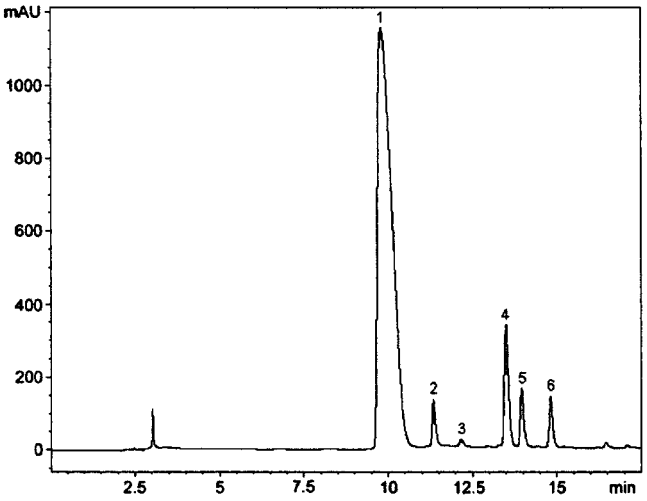


**Figure 1.** HPLC profile of anthocyanins in evergreen blackberry. Compounds: **1**, cyanidin 3-glucoside; **2**, cyanidin 3-rutinoside; **3**, unknown; **4**, cyanidin-containing xylose; **5**, cyanidin 3-glucoside acylated with malonic acid; **6**, cyanidin 3-dioxalylglucoside.

*(C) Organic Acid Analysis (System IV). (1) Apparatus.* The same equipment as described for systems I and II was used.

*(2) Columns, Mobile Phase, and HPLC Conditions.* Organic acid analysis was performed using an analytical scale (250 mm × 4.6 mm i.d.) Spherisorb ODS-2 reversed phase column with a particle size of 5 μm (Alltech Associates, Deerfield, IL), fitted with an ODS-10 (40 × 4.6 mm i.d.) Micro-Guard column (Bio-Rad Laboratories, Richmond, CA). Monitoring was performed at 214 and 224 nm at a flow rate of 0.6 mL/min. The mobile phase used was a phosphate buffer (27.2 g of $KH_2PO_4$/L), adjusted to pH 1.9 with concentrated orthophosphoric acid. Solvents and samples were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp.).

**Electrospray Mass Spectrometry (MS).** Low-resolution MS was done using electrospray MS. The instrument was a Perkin-Elmer SCIEX API III+ mass spectrometer (Norwalk, CT) equipped with an ion spray interface (ISV = 4700, orifice voltage of 120 eV). The mass spectrometer was operated in the positive-ion mode. The purified anthocyanin was dissolved in 0.1% aqueous TFA/100% MeOH (1:1, v/v) and injected directly into the system.

**Tandem Mass Spectrometry (MS/MS).** Collision-induced dissociation (CID) of the isolated anthocyanin was carried out using argon as the target gas. The mass of the parent ion was scanned in the first quadrupole (Q1), *m/z* selected and collisionally activated in Q2, and the daughter ions were analyzed in the third quadrupole (Q3). MS/MS was performed using a collision energy set of +10 eV.

## RESULTS AND DISCUSSION

**HPLC.** The anthocyanin composition in extracts from fresh fruit as well as of evergreen blackberry (*Rubus laciniatus* Willd.) concentrate was checked by HPLC. In both commodities the pigment pattern and the spectral properties of the individual components were found to be the same. Therefore, the minor peak of interest is not an artifact formed during production and storage of the concentrate. Juice concentrate was used as the source for pigment characterization. A representative chromatogram of evergreen blackberry is shown in Figure 1 with the peak areas at 520 nm of compounds **1**−**6** accounting for 82.6, 2.4, 0.7, 7.7, 3.6, and 3.0%, respectively. Except for a minor peak (**3**), the chromatographic anthocyanin pattern was the same

as previously reported by other workers (*9*−*11*): cyanidin 3-glucoside (**1**), cyanidin 3-rutinoside (**2**), a cyanidin-containing xylose (**4**), cyanidin 3-glucoside acylated with malonic acid (**5**), and an additional acylated cyanidin-based structure (**6**). The UV−vis spectra of peaks **1**−**6** monitored during on-line HPLC showed visible absorption maxima of 515 nm with an $A_{440}/A_{520}$ ratio of 31% typical for 3-substituted cyanidin glycosides (*17*). Saponification of compound **6** yielded only cyanidin 3-glucoside (**1**), further confirming a cyanidin-based structure. These findings were consistent with the fact that blackberries exclusively biosynthesize cyanidin glycosides as primary structures (*18*). Compared to **1**, compound **6** displayed a relatively long retention time on the reversed phase system, indicating acylation. Because the typical maximum absorbance at 310−320 nm for hydroxycinnamic acid derivatives could not be observed, substitution with aliphatic acids was suspected. Identification of the acylating moiety was performed by saponification of compound **6**. By subsequent HPLC analysis of the organic acid fraction the presence of oxalic acid at 4.2 min was determined.

**Electrospray and Tandem Mass Spectrometry.** For further characterization of anthocyanins with aliphatic acid residues, positive or negative fast atom bombardment techniques have been used by a number of workers (*7*, *19*−*21*). Atmospheric pressure ionization was applied to the identification of acetylated anthocyanins from *Vitis vinifera* L. (*22*), and electrospray ionization techniques were used to characterize malonyl derivatives of pelargonidin from *Raphanus sativus* L. (*23*). In this study, low-resolution electrospray mass spectrometry of compound **6** in the positive ionization mode at a cone voltage of 40 eV showed *m*/*z* values of 593 and 287. When the orifice voltage was increased to 120 eV, a good fragmentation was achieved, yielding *m*/*z* values of 593 [$M^+$] for the parent ion, as well as *m*/*z* values of 449 [cyanidin + glucose]$^+$ and 287 [cyanidin]$^+$ for the main daughter ions. The difference of *m*/*z* 144 [$M^+$ − cyanidin − glucose] after the first fragmentation, together with the findings from the organic acid analysis, indicated two oxalic acid moieties. An intermediate fragment at *m*/*z* 521 due to the mono-oxalyl derivative could not be detected at any of the orifice voltages applied. This fact indicated an oxalic acid dimer rather than two oxalic acid residues attached to two different sites of the glucose. These findings were further substantiated by comparison with the fragmentation behavior of anthocyanin malonyl derivatives, when either one acyl moiety was removed from the whole pigment alone or together with a glucose as a hemiester (*24*, *25*). However, when dimalonylated structures were studied, the two acyl residues were attached to positions 3″ and 6″ of the glucose, respectively (*25*). To the best of our knowledge, two aliphatic acyl moieties in series have never been reported before.

Further unexpected minor fragments were observed at *m*/*z* 607, 177, 133, and 89 in the MS analysis. The *m*/*z* value of 607 [M + MeOH − $H_2O$]$^+$ was due to the formation of a methyl ester in the MS solvent containing TFA and methanol. The resulting methyldioxalyl ester (M*) was detected at *m*/*z* 177 [M* + H]$^+$ together with the fragments after consecutive loss of $CO_2$ leading to *m*/*z* values of 133 and 89. This decarboxylation was additionally confirmed by MS/MS of the ions at *m*/*z* 177 and 133, the first leading to fragments at *m*/*z* 133 and 89 and the latter yielding *m*/*z* 89. Because no masses corresponding to two methylmono-oxalyl esters were detected, this again proved the attachment of an oxalic acid dimer to the cyanidin 3-glucoside structure. Re-examination of the sample by HPLC showed an additional peak eluting after compound **6** at 20.2 min indicative of methyl ester formation (*9*, *26*) typical for dicarboxylic acids.

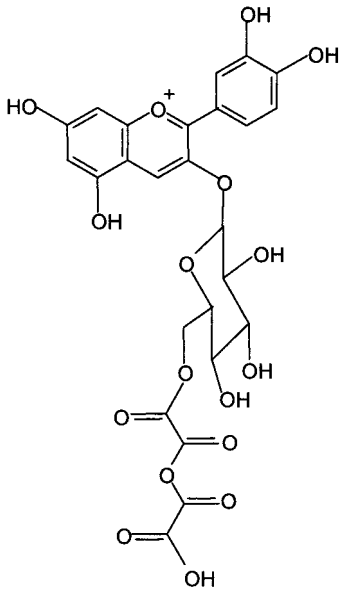


**Figure 2.** Structure of cyanidin 3-(6″-dioxalylglucoside).

**Partial Acid Hydrolysis.** Aqueous acid hydrolysis of semipurified anthocyanins from evergreen blackberry yielded cyanidin, accompanied by cyanidin 3-glucoside, the presence of the latter indicating partial hydrolysis (*9*). Andersen and Fossen (*27*) found substituents in the 6″-position of the sugar to be more labile during partial acid hydrolysis compared to the 3″-position. The stability difference of these two glycosidic bonds would have caused a more resistant 3″ intermediate, yielding an additional peak in the HPLC profile eluting before compound **6**. Because this was not observed in this study, acylation at two different sites of the glucose in compound **6** could again be excluded.

Additional evidence for the nature of the acyl moiety was given by Sapers et al. (*9*) after partial hydrolysis of compound **6** in ethanolic HCl, when a less polar structure resulting in a longer retention time was observed. The formation of this artifact was due to an esterification of the free carboxylic function and therefore indicated the presence of a terminal dicarboxylic acid. It is worth noting that extraction or storage in alcoholic media generally leads to the formation of labile esters when catalyzed by the presence of strong acids (*25*, *26*) as was substantiated by the MS analysis of compound **6** in this study. Anthocyanin pigments substituted with zwitterionic aliphatic acids are particularly labile in acidic alcoholic media, a fact that has to be considered during their extraction, purification, and storage. On the other hand, this derivatization allows distinction between mono- and dicarboxylic acids attached to the anthocyanin glycoside as was shown in the MS analysis of compound **6**.

Considering the above-mentioned results, we tentatively assign the structure of compound **6** as cyanidin 3-dioxalylglucoside. However, the exact site of acylation at the glucose moiety could not be determined by the data obtained. The proposed structure for the novel pigment is shown in Figure 2. The site of esterification is illustrated as being on 6″, because this has been shown to be the preferred site for acylation (*28*). NMR studies, which require substantial quantities of purified pigment, would be needed to fully confirm the site of acylation. Oxalic acid is an uncommon acyl residue never reported before to occur in plant families other than the Orchidaceae (*7*, *29*). This is also the first characterization of an anthocyanin structure substituted with an oxalic acid dimer.

Interestingly, the decrease of compound **6** during ripening was directly correlated to the increase of cyanidin 3-glucoside (*9*). This can be explained by the need for acylation for the anthocyanin transfer into the vacuole during pigment accumulation (*30*). Whereas evidence was given for succinyl and malonyl transport mechanisms (*31*, *32*), oxalyl transferases have not been reported so far. From a chemical point of view, the ionic attraction between the positively charged flavylium core and the free carboxylic acid may account for the stabilization of these zwitterionic anthocyanins. Natural colorants and processed foods containing significant amounts of this class of pigments would be expected to benefit from the enhanced color stability.

## SAFETY

Special attention has to be paid when toxic solutions of acetonitrile are used. Chloroform is a toxic agent, and it is explosive when mixed with acetone in the presence of strong alkali.

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