Structural and Conformational Characterization of a Stable Anthocyanin from *Tradescantia pallida*

Alan J. Baublis and M. Dolores Berber-Jiménez*

Department of Food Science, University of Illinois, Champaign-Urbana, Illinois 61801

Anthocyanins found in some plants of the Commelinaceae family have shown to be very stable compared to other commercially available natural pigments. One source of these stable anthocyanins is found in the plant *Tradescantia pallida*. Through the use of chemical hydrolysis, mass spectrometry, and ^{1}H NMR, the major anthocyanin from *T. pallida* was characterized to be 3-*O*-[6-*O*-[2,5-di-*O*-(*E*)-α-L-arabinofuranosyl]-β-D-glucopyranosyl]-7,3′-di-*O*-[6-*O*-(*E*)-feruloyl-β-D-glucopyranosyl]cyanidin.

Keywords: *Anthocyanins; tradescantia; Commelinaceae; stability pigments; structure characterization*

INTRODUCTION

The search for highly stable anthocyanins, to offer an alternative to synthetic red dyes, has led researchers to extensively study fruit and vegetable sources as well as ornamental plants. Many of the more stable anthocyanins discovered to date consist of complex chemical structures. Sophisticated polyacylated anthocyanins allow good intramolecular copigmentation upon probable sandwich-type complex formation (Dangles et al., 1993). This formation prevents the nucleophilic attack of water, preventing discoloration of the pigment. Examples of stable pigments include one from Heavenly Blue morning glories (HBA), which has six glucose units and three caffeic acid units (Kondo et al., 1987), and Ternatin D from *Clitorea ternatea*, which is a delphinidin derivative with seven glucose units, four molecules of *p*-coumaric acid and one molecule of malonic acid, (Terahara et al., 1989). Anthocyanins from the plant species *Tradescantia pallida* have been reported to also display excellent stability when compared to other anthocyanin extracts (Shi et al., 1992; Baublis et al., 1994). Some structure characterization studies have been done with this pigment (Idaka et al., 1987a; Shi et al., 1993), but complete structure elucidation is necessary to determine the stability mechanism of this pigment.

MATERIALS AND METHODS

Leaves of tradescantia (*T. pallida* cv. Purple Heart) were collected from greenhouse-grown plants (Department of Horticulture, University of Illinois, Urbana, IL) and freeze-dried immediately using a Vacudyne Freeze Drier (Chicago, IL) chamber equipped with a Copeland (Sidney, OH) pump.

Extraction of Anthocyanins. Leaves and stems (100 g) were extracted at room temperature using 3700 mL of reagent grade 3% trifluoroacetic acid (TFA) in water (v/v). The extract was filtered under vacuum through Whatman No. 2 filter paper. The concentrated filtrate was then loaded on an Amberlite XAD-7 resin column (Aldrich, Milwaukee, WI, 3 × 30 cm) washed with 1500 mL of distilled water and eluted with acetonitrile. The extract was then concentrated to dryness using a Büchi rotovap (Switzerland) connected to a Welch (Skokie, IL) high-vacuum pump at $T = 30$ °C. After evaporation, the dried crude extract was placed in a desiccator over calcium sulfate to give approximately 1 g of the tradescantia crude extract.

High-Performance Liquid Chromatography. The equipment used for HPLC consisted of a Hitachi (Tokyo, Japan) L-6200A Intelligent Pump, a Hitachi L-4500 diode array detector, and a Rheodyne (Cotati, CA) 7125 injector valve. Data were collected and processed with Hitachi Model D-6500 Chromatography Data Station software loaded on a Gateway 2000 (N. Sioux City, SD) 486DX2 personal computer. The pigments were monitored at $\lambda = 520$ nm and scanned from 300 to 600 nm. Separations were performed at room temperature, approximately 20 °C. Solvents were of HPLC grade with the exception of tetrahydrofuran (THF), which was of reagent grade. Solvents were prefiltered through a 0.45-μm nylon filter and degassed.

(a) Analytical HPLC. Analysis of the tradescantia crude extract was performed using a YMC (Wilmington, NC) S5 120A ODS-AM guard column connected to a YMC-Pack ODS-AM (4.6 × 250 mm), 5 μm particle size column. A 50 μL loop was used. Solvent A was 10% formic acid in water (v/v); solvent B was methanol–THF (1:1). The separation consisted of an isocratic elution of 15% B in A at a flow rate of 0.9 mL min^{-1}.

(b) Semipreparative HPLC. The separation was performed on the tradescantia crude extract to collect the major anthocyanin. Semipreparative HPLC was performed using a YMC-Guardpack ODS-AM (10 × 30 mm) guard column connected to a YMC-Pack ODS-AM (10 × 250 mm) column. The elution profile was the same as for the analytical separation. The flow rate was 4.2 mL min^{-1}, and an injection loop of 1.0 mL was used. The major peak was collected and concentrated to dryness under vacuum. The collected pigment underwent a second purification step: solvent A was 10% formic acid in water (v/v), and solvent B was acetonitrile. The elution profile consisted of 25% solvent B in A at a flow rate of 2.4 mL min^{-1}; the major peak was collected and evaporated to dryness. It was dissolved in a minimal amount of TFA, and the salts were precipitated with cold ethyl ether. The solid was filtered off and washed with ethyl ether. The purified pigment, 0.6 mg, was named tradescantin and used for further analysis. Multiple extractions were done as needed.

Chemical Hydrolysis of the Anthocyanin from Tradescantia. Acid hydrolysis of tradescantin was performed as reported by Shi et al. (1992a). The pigment (1.3 mg) was dissolved in 2 mL of 8 N HCl and heated at 100 °C for 10 min. The hydrolysate was then cooled to room temperature and loaded onto a pretreated OnGuard-RP (Dionex, Sunnyvale, CA) column. The column was washed with 20 mL of water to obtain the carbohydrate fraction. The aglycon and acyl groups (cinnamic acids) were eluted with 100% methanol. The two separate portions, (a) aqueous and (b) methanolic, were concentrated to dryness under vacuum. The dried carbohydrates were dissolved in 2 mL of water and then neutralized

* Author to whom correspondence should be addressed [e-mail berber@uiuc.edu; fax (217) 333-7370].

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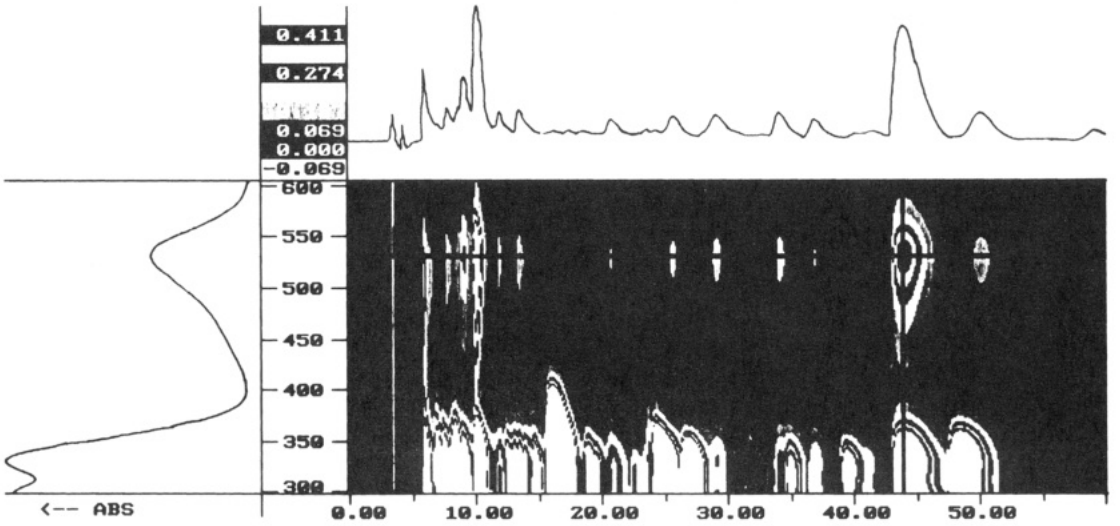

Figure 1. DAD data of the crude anthocyanin extract from *T. pallida* scanned from $\lambda = 300$ to 600 nm. The cursors at $\lambda = 532$ and RT = 44 min indicate the pigment studied: tradescantin.

with sodium bicarbonate. This sample was filtered with a Millex-HV_{13} filter (Millipore, Bedford, MA) before analysis. The methanolic fraction was dissolved in water, and the cinnamic acids were extracted with ethyl ether. The ethyl ether portion was evaporated to dryness under N_2 gas.

(a) Carbohydrate analysis was performed using a Dionex gradient pump, a Dionex pulse amperometric detector, a Hitachi AS-2000 autosampler with a Spectra-Physics (San Jose, CA) SP4290 integrator, and Hitachi Model D-6500 Chromatography Data Station software loaded on a Gateway 2000 486DX2 personal computer for collection of data. Carbohydrates were separated with a Dionex CarboPac PA1 column (250 × 4 mm) using an isocratic elution of 15 mM sodium hydroxide. External carbohydrate standards (glucose, xylose, arabinose, and galactose) were run along with the unknown sample.

(b) Analysis of cinnamic acids was performed using the same analytical RP-HPLC instrumentation as before. The solvent system used was a modification of a method from Pussayanawin and Wetzel (1987): solvent A, 10% formic acid in water (v/v); solvent B, methanol. Separation was obtained by an isocratic elution of 20% B in A at a flow rate of 0.9 mL min^{-1} which was monitored at $\lambda = 280$ nm. An external standard solution of cinnamic acids including ferulic, caffeic, *p*-coumaric, and sinapic acid (Sigma Aldrich Chemical Co., St. Louis, MO) was run along with the hydrolysate.

Enzymatic Hydrolysis of Anthocyanin from Tradescantia. Two methods were used.

(a) Analysis of Acids and Carbohydrates. Half a milligram of tradescantin was dissolved in 2 mL of distilled water in a vial. About 0.1 mg of β-glucosidase (from almonds, 4−12 units/mg, Sigma Chemical Co., St. Louis, MO) was added. The vial was capped and covered with aluminum foil at 20 °C for 3 days before analysis. Analysis of cinnamic acids was performed using the same analytical RP-HPLC equipment as stated before with the exception of the column and solvents. A YMC S5 120A ODS-AM guard column connected to a Whatman Partisil ODS-3, 5 μm (250 × 4.6 mm), column was used to separate the cinnamic acids. The solvent system used was a modification of a method from Pussayanawin and Wetzel (1987): solvent A, sodium citrate buffer (pH 5.4); solvent B, methanol. Separation was obtained by an isocratic elution of 23% B in A at a flow rate of 1 mL min^{-1}, which was monitored at $\lambda = 280$ nm. Carbohydrate analysis was performed as in the previous section.

(b) Pigment Analysis. Approximately 0.5 mg of tradescantin was dissolved in 3 mL of pH 4.5 buffer solution. β-Glucosidase (0.03 mg) was added, and the sample was allowed to sit covered at room temperature for 6 days. The hydrolyzed pigment was then collected using semipreparative HPLC (first elution profile). The hydrolyzed pigment was collected and evaporated to dryness. The dried pigment was dissolved in 10% TFA-*d* in CD_3CN and transferred to an NMR tube for analysis.

Table 1. Molecular Ion of Pigment Obtained from Various Ionization Techniques

ionization technique	M^+ (m/z)
low-resolution FAB	1609.8
high-resolution FAB	1609.443900
electrospray	1609.9

Table 2. Fragments Identified from the MS/MS Spectra

fragment (m/z)	molecule(s)
177.0	ferulic acid
287.0	cyanidin
625.2	cyanidin + glucose + ferulic
963.3	cyanidin + 2 glucoses + 2 ferulics
1271.5	cyanidin + 2 glucoses + arabinose + 3 ferulics
1609.5	cyanidin + 3 glucoses + arabinose + 4 ferulics

Controlled Acid Hydrolysis. A partial hydrolysis of the molecule was performed to simplify the proton NMR spectra. The pigment (ca. 0.5 mg) was dissolved in 1 mL of 30% TFA−CH_3CN and allowed to sit overnight at room temperature. The major product of the hydrolyzed sample was separated using semipreparative HPLC (second elution profile) and then analyzed using ^1H NMR.

Mass Spectrometry of Anthocyanin from Tradescantia. Low-resolution MS was done using fast atom bombardment (FAB) MS. The instrument used was a FISONS VG Analytical ZAB-SE using a dithiothreitol−dithioerythritol (3:1) matrix. High-resolution MS and tandem MS were performed using a FISONS VG 70-SE-4F in the same matrix. The collision gas used for tandem MS was argon. Electrospray MS was also performed on the major pigment using a VG Quattro instrument in a matrix of water, formic acid, acetonitrile, and methanol.

NMR of Anthocyanin from Tradescantia. All NMR experiments performed were on a Varian U-400 spectrometer equipped with a QUAD probe. The dried purified sample was dissolved in a solution of 10% TFA-*d* in CD_3CN, 10% TFA-*d* in CD_3OD, or 10% TFA-*d* in dimethyl-d_6 sulfoxide (Aldrich, Milwaukee, WI), depending upon experiment to be performed, and then transferred to a 528-PP NMR (Wilmad, Buena, NJ) tube for analysis. Chemical shifts were recorded as parts per million (ppm) downfield from the solvent as a reference.

RESULTS AND DISCUSSION

Chromatography. Analysis of the tradescantia crude extract was done with HPLC. Figure 1 shows the DAD data of the analytical separation of the extract at

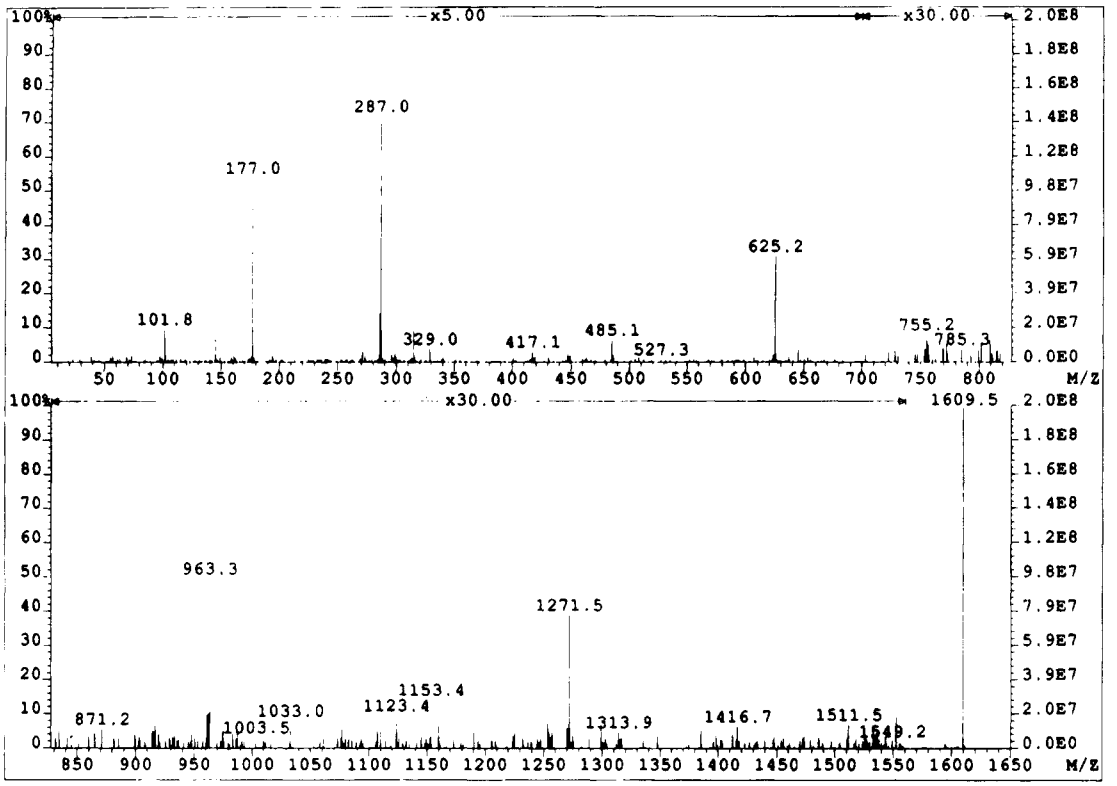

Figure 2. MS/MS spectra of tradescantin.

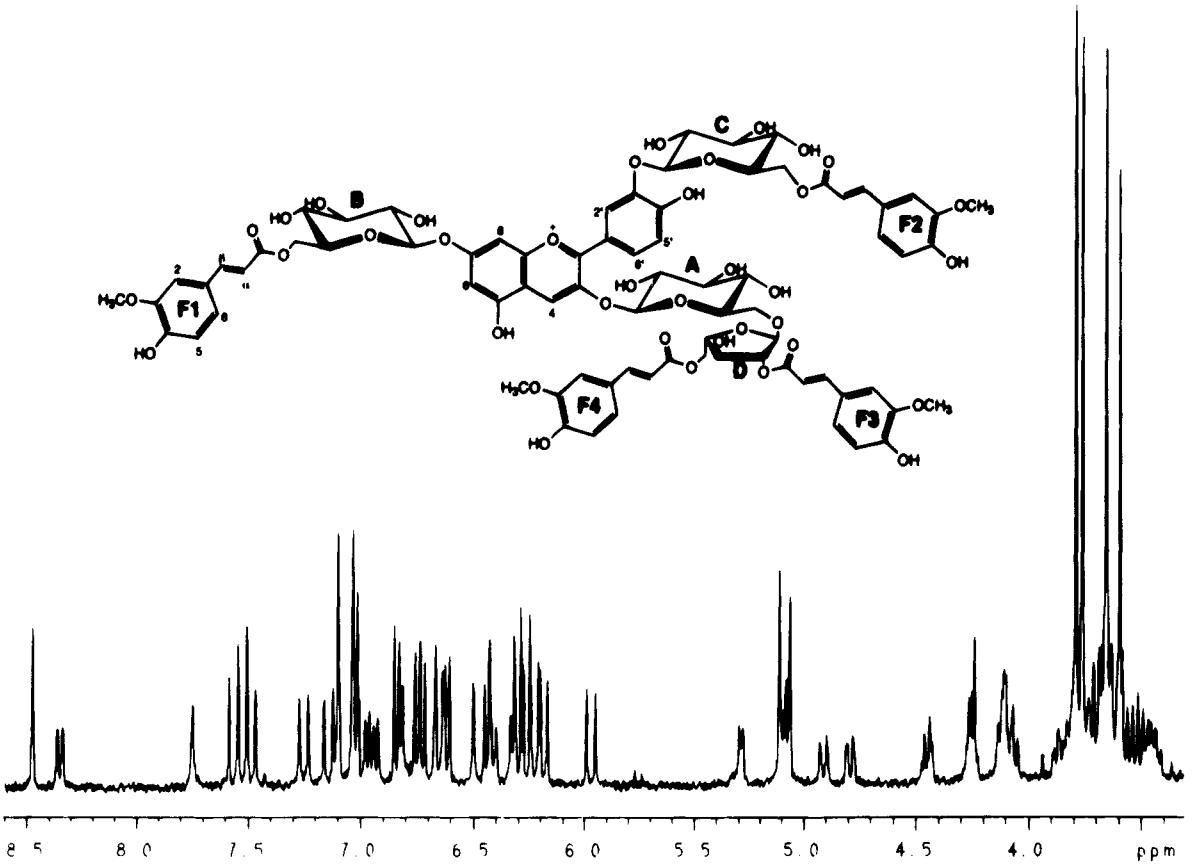

Figure 3. ^{1}H-NMR spectra of tradescantin in 10% TFA-d–CD_3CN (19 °C, 400 MHz). Letters indicate carbohydrate and ferulic acid substituents.

UV and visible wavelengths. Tradescantin is indicated by the two cursors and was the pigment characterized in this work. The pigment was first collected using the same solvent system as the analytical run followed by a second run with 10% formic acid and acetonitrile. The second run was necessary to separate a closely absorbing UV compound.

Hydrolysis. Enzymatic hydrolysis of tradescantin

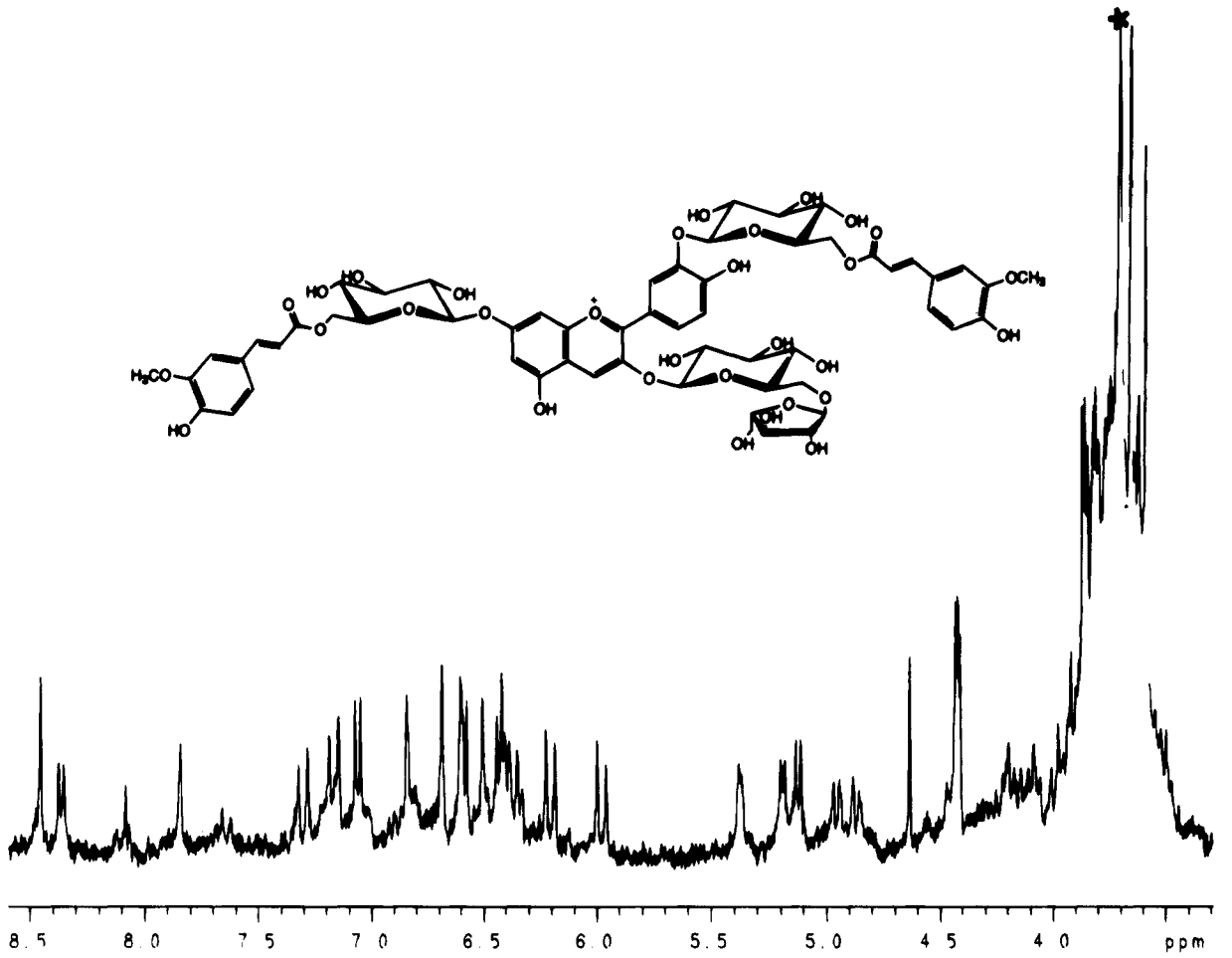

Figure 4. ^{1}H-NMR spectra of an acid-hydrolyzed fragment of tradescantin in 10% TFA-*d*−CD_3CN (19 °C, 400 MHz). The asterisk indicates an impurity.

was followed by HPLC analysis of the carbohydrates and cinnamic acids. Results from these experiments showed the presence of ferulic acid and no carbohydrates. The tradescantin NMR spectra only showed loss of one set of vinylic protons and partial hydrolysis of both upfield methoxy signals. All of the anomeric protons (four) were still present. The hydrolysis product identified was tradescantin minus a ferulic acid only, and it was believed to be due to side esterase activity in the enzyme preparation.

Complete acid hydrolysis was performed on tradescantin to determine acyl groups present. Only ferulic acid was found. Analysis of the carbohydrates showed glucose and arabinose. Quantitative analysis was not done because of the hydrolysis step. Both the cinnamic acids and carbohydrates showed decomposition under the conditions used.

UV Spectra. Data from the diode array detector were used to determine the number of aromatic group substituents. It was reported by Idaka et al. (1987) that the number of acyl groups could be estimated using Harborne's rule. The E_{UV}/E_{vis} for tradescantin was 0.065/0.029 = 2.2. Multiplying by a conversion factor of 2 gives approximately four acyl groups.

The measured E_{440}/E_{max} of tradescantin was 25%, indicating that the C-5 position of the cyanidin was free (Harborne, 1958).

Mass Spectra. Table 1 summarizes the results obtained from MS using different ionization techniques. The molecular weight of tradescantin was 1609.5. Tandem MS (MS/MS) was also used to specifically fragment the molecular ion peak, therefore revealing fragments of the single molecule that are not found in the FAB ionization technique (Strack et al., 1992). Figure 2 shows the MS/MS spectra obtained when the molecular ion peak (1609.5 *m/z*) is selectively ionized. The major fragments were identified and are summarized in Table 2. The results from MS/MS agreed with the high-resolution FAB, confirming the molecular formula of $C_{78}H_{81}O_{37}$.

The components of tradescantin were determined from the results presented to this point. NMR was used to determine how these fragments were arranged, types of bonds, configurations, and ring sizes for the complete structure.

NMR Analysis. Different solvents were tried to optimize the NMR spectra. Ten percent TFA-*d* in CD_3CN, DMSO-d_6, or CD_3OD was used. The molecule behaved differently depending on the solvent used. The spectra of the pigment in 10% TFA-*d*−CD_3CN had the advantage of a good separation of signals in the aromatic region, while the carbohydrate area was not well resolved. Another disadvantage to 10% TFA-*d*−CD_3CN is that difference NOE experiments were not possible due to the low T_1 value of the molecule in this solvent. The 10% TFA-*d*−DMSO-d_6 sample had the advantage of very large NOEs at room temperature, but the resolution of the signals, in both the aromatic and carbohydrate regions, was poor and made interpretation difficult. The 10% TFA-*d*−CD_3OD showed a very good separation of signals in the carbohydrate region of the spectra, while the aromatic area was slightly over-

Table 3. Chemical Shifts, Coupling Constants, and Multiplicities of Protons Identified in the Major Pigment of Tradescantia: A, the Chromophore; B, the Acyl Residues; and, C, the Carbohydrates of Tradescantia Major Pigment

A. Chromophore Protons
(10% TFA-d–CD_3CN at 19 °C, 400 MHz)

^1H	δ (ppm)	J (Hz)
4	8.47s	
6	6.84d	1.2
8	6.70d	1.2
2′	7.76d	2.0
5′	7.02d	8.8
6′	8.34dd	8.8, 2.0

B. Ferulic Acid Protons
(10% TFA-d–CD_3CN at 19 °C, 400 MHz)

^1H	F1	F2	F3	F4
α	6.22d	5.90d	6.27d	6.20d
β	7.35d	7.18d	7.50d	7.49d
2	6.62d	6.50d	7.06d	7.02d
5	6.58d	6.44d	6.75d	6.72d
6	6.40dd	6.34dd	6.96dd	6.92dd
OCH_3	3.66s	3.58s	3.79s	3.76s

C. Carbohydrate Protons
(10% TFA-d–CD_3OD, 400 MHz)

CHO-^1H	δ	J (Hz)
A-1	5.12d	7.9
A-6a	4.49dd	12, 2
B-1	5.36d	7.9
B-6a	4.91dd	10, 2
C-1	5.18d	7.9
C-6a	5.19dd	12, 2
D-1	5.14s	
D-2	5.15d	2.5
D-3	4.06dd	6.0, 2.5
D-4	4.25ddd	6.0, 5.0, 2.5
D-5a	4.48dd	12, 2.5
D-5b	4.23dd	12, 5.0

lapped. NOE experiments were possible in 10% TFA-d–CD_3OD at room temperature, but better results are obtained at low temperature. However, water protons exchange with CD_3OD, resulting in a large water peak at approximately 5.3 ppm for this spectra. The water peak covered various signals in the carbohydrate region and moved depending upon concentration and temperature.

Two NMR solvents were used for structure elucidation: 10% TFA-d–CD_3OD for assignment of signals in the carbohydrate region as well as attachment of carbohydrates through NOE experiments and 10% TFA-d–CD_3CN to assign both protons attached to the chromophore and protons associated with acyl groups.

Figure 3 shows the proton spectra of tradescantin in 10% TFA-d–CD_3CN. The peak assignments were done using chemical shifts, coupling constants, and ^1H–^1H COSY. The signal for H-4 of the anthocyanidin nucleus had the largest chemical shift at 8.47 ppm. Table 3A summarizes all chemical shifts and coupling constants of the chromophore protons. The chromophore was cyanidin, which agrees with the MS results. The remaining signals in the aromatic proton region corresponded to the four molecules of ferulic acid. The coupling constants between the α and β protons were 16 Hz, which indicates that the acids have an E configuration. The partially hydrolyzed sample (Figure 4) simplified the aromatic region of the spectra since two molecules of ferulic acid were lost. The remaining two ferulic acid molecules were identified in the complete spectra of the molecule. Table 3B summarizes the chemical shifts for the protons of the four ferulic acid molecules. The coupling constants for all of the protons of the acid molecules were as follows: $J_{\alpha-\beta} = 16$ Hz, $J_{2-6} = 2$ Hz, $J_{5-6} = 8$ Hz.

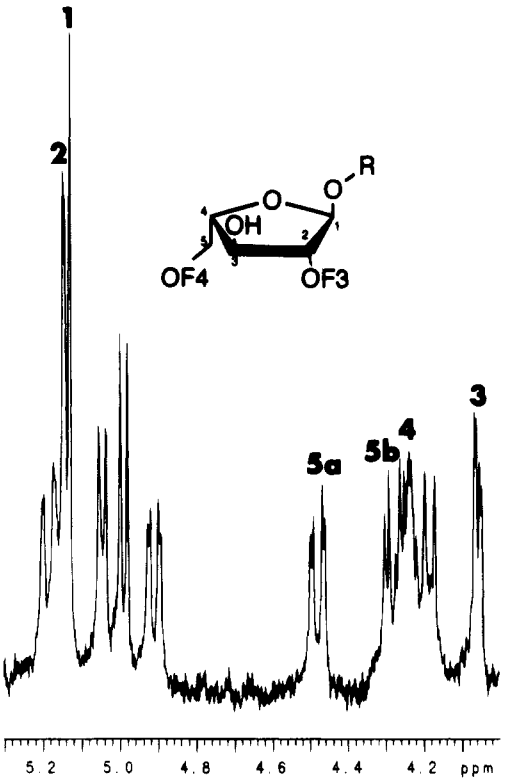

Figure 5. ^1H-NMR spectra of the carbohydrate region of tradescantin in 10% TFA-d–CD_3OD (19 °C, 400 MHz).

Figure 5 shows the carbohydrate area of the proton spectra of tradescantin in 10% TFA-d–CD_3OD. Four anomeric protons were observed in the region around 5 ppm, indicating the presence of four carbohydrates. The attachment of the carbohydrates to the chromophore was detected through the use of NOE difference spectroscopy. Figure 6 shows the NOE of anomeric protons. Irradiation at A-1, B-1, and C-1 resulted in negative NOE signals at H-4 (−20%), H-6 (−9.9%), and C-3′ (−14%) of the chromophore, respectively. Therefore, carbohydrate A was attached at the C-3 position, B at C-7, and C at C-3′ of the cyanidin. When carbohydrate D was irradiated, no NOE in the aromatic region was observed. The NOE results obtained correlated closely to those reported by Yoshida et al. (1991) for a cyanidin molecule with glucose attached at the C-3, C-7, and C-3′ positions. The coupling constant of these anomeric protons was 7.9 Hz, indicating β anomers. All of the corresponding protons were determined to be in the D-glucopyranosyl forms.

The identification of the remaining carbohydrate (D) was determined also using ^1H–^1H COSY data. This carbohydrate was concluded to be α-L-arabinofuranosyl on the basis of chemical shifts and coupling constants of the protons and reported data from Idaka et al. (1987b). Figure 5 shows the assignment of protons of carbohydrate D. The chemical shifts for the identified carbohydrate protons are presented in Table 3C.

The NMR spectra were examined more closely to determine type of bonding. There were only two glucosides substituted by a ferulic acid in position 6 as seen by the downfield shift of these protons (protons between 4.8 and 4.9 ppm in Figures 3 and 4). The spectra of

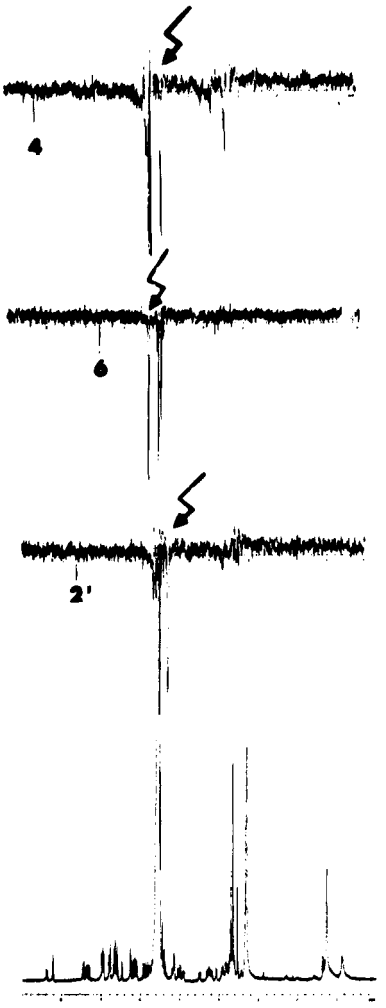

Figure 6. NOE spectra of anomeric protons A (A-1), B (B-1), and C (C-1) in 10% TFA-*d*−CD_3OD (19 °C, 400 MHz).

the controlled hydrolyzed sample (Figure 4) showed that the two ferulic acid molecules remaining, F1 and F2, were those absorbing upfield (β protons around 7.2 ppm). It was concluded then that positions C-7 and C-3′ of tradescantin were substituted with glucose−6-*O*-ferulic acid units. This was also confirmed by the MS/MS data, in which the fragment at 1271.5 *m/z* indicates the loss of a glucose unit and a ferulic acid unit.

Some of the protons for the arabinose unit were shifted upfield upon hydrolysis (Figure 4), while the third glucoside remained unchanged. The arabinose unit was determined to be glycosidically linked to 6-OH of the glucose attached to C-3 of the chromophore. This linkage was ascertained on the basis of the chemical shift of H-6a of the glucose molecule A and also on data reported by Idaka et al. (1987b). It was discovered that two ferulic acid molecules (F3 and F4) were attached to arabinose at positions C-2 and C-5 because of the chemical shift of these protons. These results were supported by the MS/MS data by the large fragment observed, which corresponds to loss of an arabinose, a glucose, and two ferulic acid units. These data indicated that the arabinose was attached to the glucose unit at position C-3 and that two ferulic acids were attached to the arabinose.

The two ferulic acid units attached to the glucose at C-7 and at C-3′ (F1 and F2) were differentiated from one another on the basis of chemical shifts. The protons of the ferulic acid attached to the glucose at C-7 are shifted downfield compared to the ferulic acid attached to C-3′. This difference in chemical shifts correlated to the difference in chemical shifts observed for the anomeric carbons for these carbohydrates. Therefore, it was concluded that F1 was attached to the glucose at C-7 and F2 was attached to the glucose at C-3′.

Analysis of the enzyme-hydrolyzed pigment showed only the nonselective cleavage of F3 or F4 in the arabinose unit. The three glucosidic bonds were intact.

CONCLUSIONS

The structure of the major anthocyanin in *T. pallida* was determined to be 3-*O*-[6-*O*-[2,5-di-*O*-(*E*)-feruloyl-α-L-arabinofuranosyl]-β-D-glucopyranosyl]-7,3′-di-*O*-[6-*O*-(*E*)-feruloyl-β-D-glucopyranosyl]cyanidin. The complete structure of tradescantin is presented in Figure 3. This is only the second reported anthocyanin with an acylated arabinose more than one unit away from the chromophore. The other anthocyanin, zebrinin, is found in the plant *Zebrina pendula*, which is in the same genera, *Commelina*, as tradescantia. The similarity between the two pigments may indicate a taxonomic relationship.

It has already been reported by Brouillard (1981) that the outstanding stability of the *Zebrina* anthocyanin is due to intramolecular copigmentation. This conclusion can be extended to tradescantin since their structures are the same except for the type of acyl groups attached. Zebrinin is acylated with caffeic acid, while tradescantin is acylated with ferulic acid. The basis for the increased stability is because these anthocyanins do not form any pseudobase and/or chalcone (Brouillard, 1981). Another observation made was that the *Zebrina* anthocyanin has a high acidity constant. It is the high acidity constant coupled with the lack of formation of the pseudobase and chalcone that he ascribes as the origin of the exceptional color stability of structurally related pigments.

Since the hydration reaction, which is so easy with ordinary anthocyanins, does not occur at all, there must be some unusual structural factor preventing the nucleophilic addition of water to the pyrylium ring (Brouillard, 1981). Brouillard suggested that the stability of *Zebrina* anthocyanins is a result of the aromatic residues of the acylated groups interacting with the positively charged pyrylium ring in such a way that the reactivity of the carbon at position 2 with nucleophilic reactants is greatly diminished. It was later postulated by Brouillard (1982) that the acyl groups are positioned in a way that one is above the chromophore and one is below. Dangles (1993) suggested that the stability of an anthocyanin is due to a spacer which probably allows a good copigmentation interaction resulting in a color stability.

Tradescantin displays superior stability because of the structural conformation. It possesses two 6-*O*-(*E*)-feruloyl-β-D-glucopyranosyl units and one 2,5-di-*O*-(*E*)-feruloyl-α-L-arabinofuranosyl unit, allowing probable sandwich-type complex formation. The data shown indicate that B-ring substitution and the right carbohydrate spacer are keys to the highly stable nature of tradescantin.

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