JOURNAL OF NATURAL PRODUCTS

Article

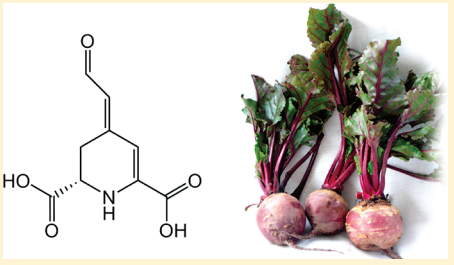
pubs.acs.org/jnp

Purification and Antiradical Properties of the Structural Unit of Betalains

Fernando Gandía-Herrero,* Josefa Escribano, and Francisco García-Carmona

Departamento de Bioquímica y Biología Molecular A, Unidad Docente de Biología, Facultad de Veterinaria, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain

ABSTRACT: Betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid] is a naturally occurring compound that is normally found condensed with amino acids, amines, cyclo-DOPA, and cyclo-DOPA derivatives to form the betalains. Betalains are the pigments responsible for the yellow to violet color of the fruits and flowers of plants belonging to the order Caryophyllales. Betalamic acid is the structural feature common to all of these pigments and contains the electron resonance system responsible for the spectroscopic properties. Betalamic acid was purified by chromatography and identified by UV−vis spectrophotometry and ESI mass spectrometry. The antioxidant and free radical scavenging capacities of betalamic acid were assessed using the FRAP and $ABTS^{\cdot+}$ radical assays. A pK_a of 6.8 was found for the deprotonation equilibrium involved in the nucleophilic activity of betalamic acid; this pK_a explains the observed pH effect on the free radical scavenging capacity of these pigments.



Betalains are water-soluble nitrogen-containing pigments that are present in the fruits and flowers of most plant species belonging to the order Caryophyllales.[1] These compounds are also present in fungi, such as *Amanita*[2] and *Hygrocybe*.[3] More than 50 natural betalains have been described[4] and are divided into the violet betacyanins and the yellow betaxanthins. These exhibit absorption maxima around 536 and 480 nm, respectively. The structural identification of betalains was first performed in the early 1960s. Wyler et al.[5] identified the betacyanin betanidin as the immonium derivative of betalamic acid condensed with cyclo-DOPA. Piattelli et al.[6] characterized indicaxanthin, the first betaxanthin identified, as the immonium derivative of betalamic acid condensed with L-proline.

Other studies have revealed the existence of betaxanthins that are derived from different amino acids and amines and the existence of complex betacyanins that have sugar moieties incorporated into their structures.[1] Betalamic acid is the pivotal molecule for the formation of the great variety of the betalain pigments. Figure 1 shows the structures of betalamic acid and its derivatives.

Although the biosynthetic pathway of betalains was partially elucidated in 1981,[7] reactions involving pigment transformation were not characterized until recently.[8] The formation of betalamic acid starts from the amino acid tyrosine, which is hydroxylated by the tyrosinase enzyme to form 3,4-dihydroxyphenylalanine (DOPA).[9] DOPA is then transformed into a 4,5-secodopa intermediate by the enzyme dioxygenase.[10]

Betalains can be found in a variety of species and tissues, but red beet (*Beta vulgaris*) roots and the fruits of cacti belonging to the genus *Opuntia* (mainly *Opuntia ficus indica*) are the best known edible sources of betacyanins and betaxanthins. The color is primarily due to the betacyanin betanin in beet root[11] and to the betaxanthin indicaxanthin in *Opuntia* fruits.[12]

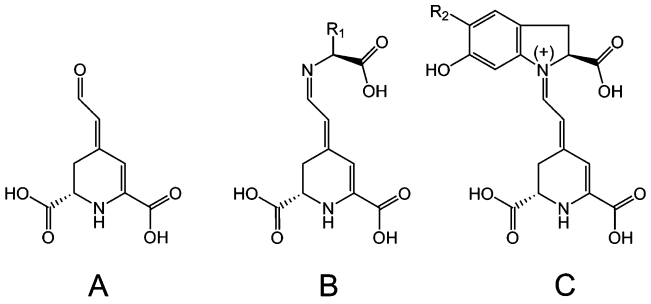


Figure 1. General structures of betalamic acid (A), betaxanthins (B), and betacyanins (C). R^1 is any lateral residue present in amines or amino acids. R^2 is −OH (betanidin), glucose (betanin), or a complex sugar residue.

Betanin-containing beet root extracts are used to give a pink or violet color to foods and beverages as the additive 73.40 in the 21 CFR section of the Food and Drug Administration (FDA) in the United States and under the E-162 code in the European Union. New colorants containing betaxanthins have also been proposed.[13] Betalamic acid in its free form has been reported to be a natural component of the fruits of *Opuntia ficus indica*.[14]

The first investigations demonstrating the radical scavenging capacity of betalains were performed with the pigments present in beet root.[15] Subsequent research revealed the existence of an intrinsic activity present in all betalains that is modulated by structural factors.[16−19] Furthermore, very low concentrations of dietary betanin have been shown to inhibit skin and liver tumor formation in mice[20] and to protect mice from the effects of γ radiation.[21] In humans, the plasma concentrations of betalain

Received: December 6, 2011
Published: May 29, 2012

after ingestion are sufficiently high that this compound is incorporated into LDL and red blood cells, where it can protect the cells from oxidative damage and hemolysis.[22,23] There has been no prior evidence of the possible biological activity of free betalamic acid.

The high solubility of betalains in water means that they can be extracted from vegetable sources in buffered aqueous solutions or purified water.[15,24] However, varying amounts of miscible organic solvents, such as EtOH,[25] MeOH,[26] and acetone,[27] are typically added to the extraction solution. Other methods for pigment extraction include the application of pulsed electric fields to fresh sections of vegetable material.[28] Betalamic acid can be synthesized in vitro using a process that involves multiple steps but gives low yields.[29] This compound can also be obtained from the degradation of betanin (betanidin-5-*O*-β-glucoside) by alkaline hydrolysis.[30] In this case, the presence of the other degradation product, cyclo-DOPA-glucoside, drives the reverse reaction, yielding the original pigment.[31] Further acidification of the hydrolysis medium and EtOAc-mediated extraction of the resulting mixture, followed by solvent evaporation and residue filtration, yield betalamic acid.[32] Despite low yields, this method has been successfully used to produce stable betaxanthins.

Betalains exhibit high antioxidant activity and free radical scavenging capacity. However, the activity of the structural unit common to all of these pigments has not been evaluated, and a suitable procedure to obtain and purify this compound in sufficient amounts has not been developed.

RESULTS AND DISCUSSION

Betalamic Acid Purification. Betalamic acid may be obtained through the alkaline hydrolysis of betanin by increasing the solution pH above 11.0. Degradation may be followed by a change in color from violet (betanin, λ_m= 536 nm) to yellow (betalamic acid, λ_m = 424 nm). However, the presence of any amino acid or amine, including cyclo-DOPA-glucoside, in solution with betalamic acid will drive condensation at moderate pH values via a Schiff reaction, which yields the corresponding imines.[33]

Hence, the Schiff reaction must be limited to obtain betalamic acid in its free form.[34] In our study, betalamic acid was chromatographically purified from the amine moiety of the betalain structure before the reaction could occur, obviating the need to acidify the reaction medium. Anionic exchange chromatography was performed on a trimethyl ammonium matrix with sample injection at the hydrolysis pH (>11.0). A phosphate buffer with a low ionic strength was used as the mobile phase after the sample was injected, followed by a gradient of NaCl to promote betalamic acid elution. Betalamic acid interacts with the cationic matrix at pH 6.0, most likely due to the two deprotonated carboxylic acid groups in the molecule.

Two different column volumes were used to evaluate the possibility of scaling up the purification process. Figure 2 shows two typical elution profiles for the purification of betalamic acid. Figure 2A shows the elution process for a 1 mL column (protocol A), and Figure 2B shows the data obtained for a 5 mL column (protocol B). The yields obtained were 86% of the starting material, as determined by spectrophotometry. This is a significant improvement over the previous protocols where the separation of betalamic acid from the amine moiety of the betalain pigment was achieved with low yields by EtOAc extraction after acidification.[32]

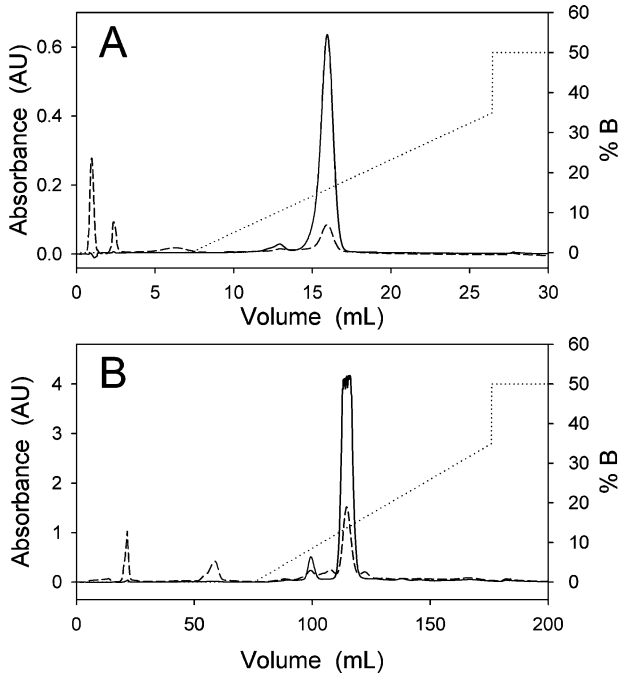


Figure 2. Purification of betalamic acid. (A) Purification protocol A, described in the Experimental Section, was followed after the injection of 100 μL of 250 μM betanin hydrolysis medium. (B) Scaled-up purification protocol B was followed after the injection of 10 mL of 250 μM betanin hydrolysis medium. The absorbance was followed at λ = 424 nm (−) and λ = 280 nm (---). The addition of solvent B (%) is also shown (···).

The aim of the protocol described herein was to separate betalamic acid from any free amine present in the hydrolysis medium to prevent the formation of imines via the Schiff reaction and to obtain pure betalamic acid.

Betalamic Acid Characterization. The betalamic acid obtained was analyzed by HPLC following a suitable protocol.[33] A typical chromatogram obtained for the analysis of the fractions purified by ionic exchange is shown in Figure 3A. The peak corresponding to betalamic acid had a retention time of t_R = 14.59 min. This peak was the only one found using a photodiode detector, thus demonstrating the high degree of purification obtained. The maximum wavelength of the absorbance spectrum was λ_m = 407 nm in the presence of MeCN under the HPLC analysis conditions. Figure 3B shows the UV−visible spectrum for betalamic acid in H_2O; this spectrum exhibited a maximum at a wavelength of λ_m = 424 nm. The molar absorption coefficient of betalamic acid was calculated by a procedure based on the basic hydrolysis of betanin to yield betalamic acid using betanin solutions of known concentrations as a reference (ε_{536} = 65 000 $M^{-1}\cdot cm^{-1}$).[35] The value determined for the betalamic acid coefficient was ε = 27 000 $M^{-1}\cdot cm^{-1}$ at a wavelength of λ = 424 nm in H_2O. This value is consistent with the data in the literature obtained by acid hydrolysis and amino acid quantification (ε = 24 000 $M^{-1}\cdot cm^{-1}$ at λ = 424 nm).[36]

The acid obtained was also analyzed by ESIMS. Sample ionization was performed in the positive mode, and the masses were detected in the m/z range 50−600. Under these conditions, an m/z of 212 was obtained for the parent ion, which corresponds to the expected protonated form of betalamic acid $[M - H]^+$. This m/z value confirmed the proposed structure of the purified molecule. Two main

dx.doi.org/10.1021/np200950n | J. Nat. Prod. 2012, 75, 1030−1036

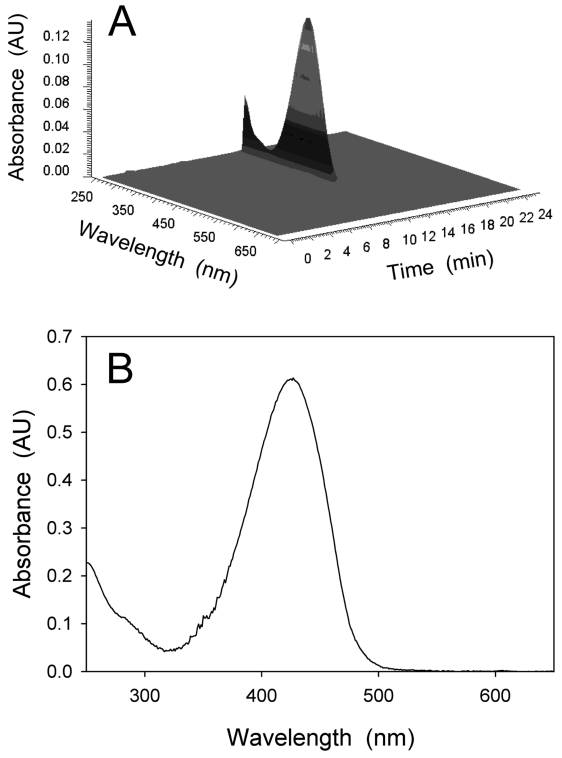


Figure 3. Analysis of betalamic acid. (A) High-performance liquid chromatography analysis of a betalamic acid sample after anionic exchange purification. A volume of 20 μL of 80 μM betalamic acid was injected. (B) UV−visible spectrum of a 25 μM betalamic acid solution in H_2O at 25 °C.

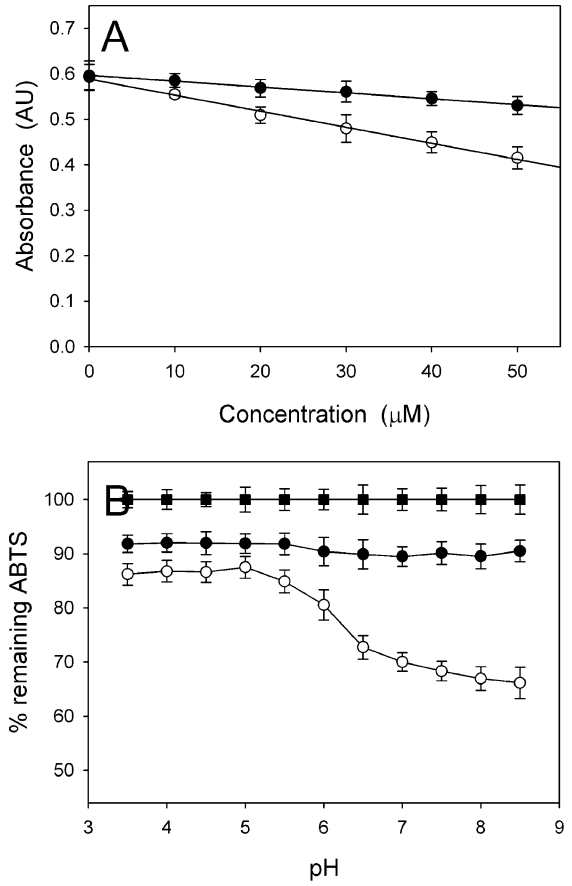


Figure 4. Free radical scavenging activity of betalamic acid. (A) Decreases in the absorbance at λ = 734 nm reflect the depletion of the $ABTS^{\bullet+}$ radical after the addition of increasing concentrations of betalamic acid (○) or Trolox (●). The starting concentration of $ABTS^{\bullet+}$ in the assay was 60 μM in a reaction medium containing 50 mM Na_3PO_4 buffer, pH 7.0. (B) Effect of pH on the free radical scavenging activity of betalamic acid. The starting concentration of $ABTS^{\bullet+}$ in the assay medium was 60 μM. Betalamic acid (○) and Trolox (●) were added at final concentrations of 50 μM to 50 mM buffered solutions with the selected pH values. Purified H_2O (■) was used in the corresponding control samples.

daughter ions of m/z 102 and 166 were obtained, with the latter most likely corresponding to the loss of one carboxylic acid group.

Free Radical Scavenging Activity of Betalamic Acid. The free radical scavenging capacity of betalamic acid was evaluated by determining the effect of this compound on solutions of the stable colored radical $ABTS^{\bullet+}$ [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)]. The $ABTS^{\bullet+}$ decolorization assay was performed by following the decrease in the absorbance at a wavelength of λ = 734 nm.[15] The effect of betalamic acid was compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a potent water-soluble antiradical derived from vitamin E that is commonly used as a reference.

Figure 4A shows the effects of the addition of six different concentrations of betalamic acid and Trolox to $ABTS^{\bullet+}$ radical solutions at pH 7.0. Increasing concentrations of both molecules were correlated with decreases in the absorbance due to the presence of the radical. The response to betalamic acid was higher. The effect of betalamic acid on the $ABTS^{\bullet+}$ radical relative to the effect of Trolox could be determined from the linear response shown. The ratio of the slopes obtained for the two compounds reflected the amount of Trolox needed to achieve the same effect as that produced by betalamic acid. The observed TEAC (Trolox equivalent antiradical capacity) value of betalamic acid was 2.7 ± 0.2. This value is equivalent to that of the antioxidant resveratrol (TEAC = 2.7),[39] which is present in grapes, and is close to the values determined for the betalain pigments with the simplest structures.[19]

pH Effect and Characterization of the Deprotonation Equilibrium. Solutions with different pHs were used in the $ABTS^{\bullet+}$ radical assay to evaluate the effect of the protonation state of betalamic acid on its free radical scavenging capacity. NaOAc was used as a buffer in solutions with pH values ranging from 3.5 to 5.5, and Na_3PO_4 was used for solutions with pH values from 5.5 to 8.5. No difference was observed in the activities measured in the two buffers at pH 5.5. As shown in Figure 4B, pH values above pH 5.5 were associated with a notable increase in the radical scavenging capacity of betalamic acid, with only basal scavenging activity observed at pH values below 5.5. In any case, the activity at more acidic pH values was higher than that exhibited by Trolox.

This marked dependence of the measured activity on pH is similar to that described for the betalain molecules, for which the effect of pH has been studied.[19] The similarity of the pH curves suggests that there exists a protonation equilibrium involving the betalamic acid moiety of the pigments, which was not demonstrated for the free acid. The protonation equilibrium for betalamic acid may result from the secondary amine group, which is conjugated via resonance with the hydroxy group that participates in the keto−enol tautomeric equilibrium of the formyl group. In this case, deprotonation

dx.doi.org/10.1021/np200950n | J. Nat. Prod. 2012, 75, 1030−1036

would yield a form with a higher nucleophilic capacity, which may explain the observed effect of pH on the free radical scavenging capacity. In addition, the nucleophilic capacity of the deprotonated form of betalamic acid would promote its nucleophilic attack of other molecules such as quinones, yielding adducts.[38,39] To observe this phenomenon, a stable 4-methylcatecholquinone was prepared from 4-methylcatechol using the catecholase activity of tyrosinase. After completion of the reaction, the medium was filtered to remove the catalyst. Figure 5A shows the evolution of 4-methylcatecholquinone over time in an assay with betalamic acid in an aqueous solution at pH 7.0. The appearance of the new product was observed as an increase in the absorbance spectrum centered at λ = 530 nm. The maximum could be detected better by subtracting the initial recording from the rest (differential spectra; Figure 5A, inset). This wavelength was used to follow the capacity of betalamic acid to attack the quinone as a function of pH. The compound 4-methylcatecholquinone was produced in the reaction media at different pH values in the presence of betalamic acid, and the existence of lag periods in the formation of the adduct was observed.

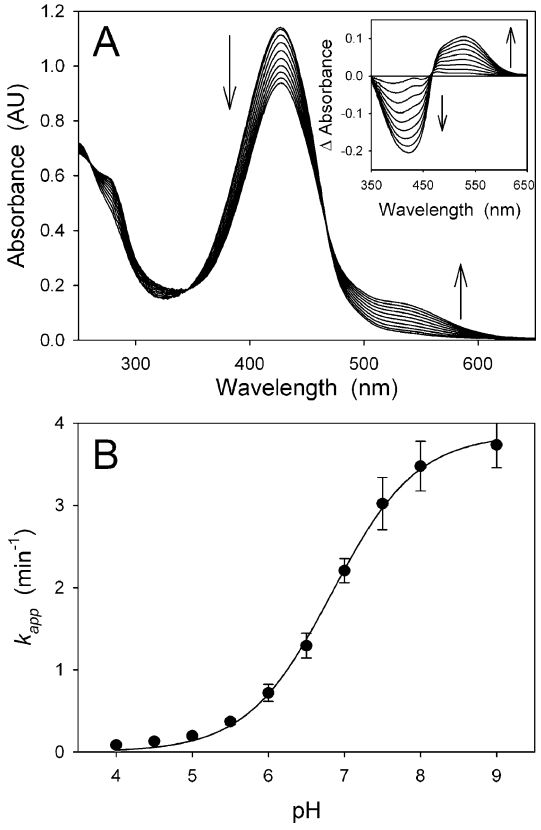
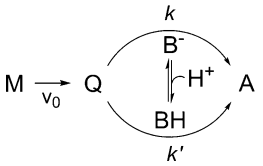


Figure 5. Nucleophilic attack of 4-methylcatecholquinone by betalamic acid. (A) Spectroscopic changes in a betalamic acid solution (45 μM) after the addition of filtered 4-methylcatecholquinone at a final concentration of 200 μM. The assay was performed in 50 mM Na_3PO_4 buffer at pH 7.0, and the spectra were recorded at 1 min intervals for 10 min. Inset: Differential spectra derived from the previous scans, subtracting the first recording from the rest. (B) Effect of pH on the nucleophilic capacity of betalamic acid measured as the apparent reaction constant (k_{app}) associated with the attack of 4-methylcatecholquinone. The medium contained 100 μM betalamic acid, 200 μM 4-methylcatechol, and tyrosinase (13 units/mL) as a catalyst in 50 mM buffered solution at the indicated pH. The reactions were followed at 530 nm. In the absence of the quinone, there was no detectable reaction.

The diagram below shows the reactions involved in the nucleophilic attack by betalamic acid of the quinones formed from 4-methylcatechol by tyrosinase:



where M is 4-methylcatechol, Q represents the corresponding quinone, BH is the protonated form of betalamic acid, B^- corresponds to the deprotonated form of the acid, and A is the adduct formed by the reaction of 4-methylcatecholquinone and betalamic acid. v_0 represents the formation rate of quinone Q from 4-methylcatechol. k and k' are the reaction constants for the nucleophilic attack of quinone Q by the deprotonated and protonated forms of betalamic acid, respectively.

The lag period in the reaction was determined at each pH, revealing a low capacity for nucleophilic attack at acidic pH values. The adduct formation reaction proceeds without a lag phase at higher pH values (results not shown). The apparent reaction constant (k_{app}) at each pH value may be calculated as the inverse of the lag period time (L) according to the following equations derived from the deprotonation equilibrium of betalamic acid and the diagram above using the stationary-state kinetic approximation:

$$K_a = \frac{[B^-][H^+]}{[BH]}$$

$$[B^-] + [BH] = [B_0]$$

$$\frac{dQ}{dt} = v_0 - (k[B^-] + k'[BH])[Q] = 0$$

$$v_0 t = [Q] + [A]$$

where K_a is the equilibrium constant for betalamic acid deprotonation and $[B_0]$ is the total concentration of the acid. The appearance of the adduct and the apparent constant for its formation (k_{app}) may be written as a function of $[H^+]$ as

$$[A] = v_0 t - \frac{v_0}{k\frac{K_a[B_0]}{K_a + [H^+]} + k'\frac{[H^+][B_0]}{K_a + [H^+]}}$$

$$k_{app} = \frac{1}{L} = k\frac{K_a[B_0]}{K_a + [H^+]} + k'\frac{[H^+][B_0]}{K_a + [H^+]}$$

At low pH values, the apparent constant for adduct formation can be simplified as $k_{app} = k'[B_0]$, whereas at high pH, $k_{app} = k[B_0]$. Figure 5B presents the values determined for the apparent constant k_{app} as a function of pH. As observed, the reaction constant was higher at high pH values than at low pH values. Thus, the curve shape was significantly affected by the deprotonation equilibrium, which is closely related to the higher free radical scavenging capacity observed when the pH was increased, as shown in Figure 4B. The nonlinear fitting of the data shown in Figure 5B to the k_{app} equation gave values for the reaction constants of k (0.0371 ± 0.0005 min^{-1}·μM^{-1}) and k' (0.0016 ± 0.0003 min^{-1}·μM^{-1}); the corresponding dissociation constant of betalamic acid was K_a = (0.1438 ± 0.0105) × 10^{-6} M. Thus, the pK_a value for betalamic acid

dx.doi.org/10.1021/np200950n | J. Nat. Prod. 2012, 75, 1030–1036

deprotonation was pK_a = 6.8. This pK_a explains the behavior of betalamic acid as a function of pH in terms of reactivity and antiradical activity. The strong antiradical capacity of betanin and its pH dependence have been explained previously in terms of betanin's electron donor capacity,[40] whereas in flavonoids, deprotonation also generates a more active phenolate anion.[41,42]

Antioxidant Activity. The antioxidant activity of free betalamic acid was evaluated through the direct reduction of Fe(III) to Fe(II). The ferric reducing antioxidant power (FRAP) assay was used, as described by Benzie and Strain,[43] to spectrophotometrically monitor the reduction at λ = 593 nm. Using Fe(II) ($FeSO_4$) solutions with known concentrations, a calibration curve was established to estimate the number of electrons involved in the reduction. Figure 6 shows the signal

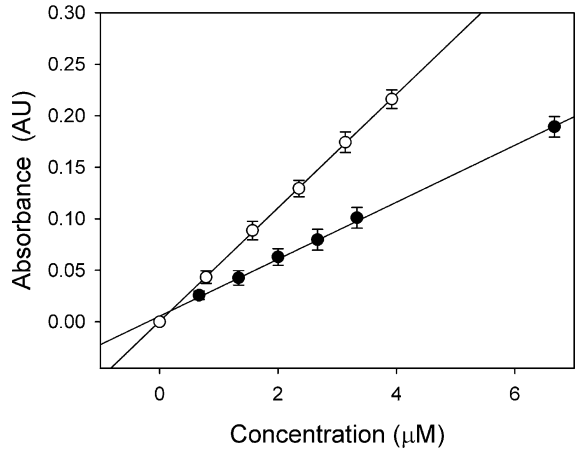


Figure 6. Antioxidant activity of betalamic acid measured by the FRAP assay. The change in the absorbance at 593 nm reflects the formation of the colored TPTZ and Fe(II) complex after the reduction of Fe(III) by betalamic acid (○). The TPTZ concentration was 741 μM, and the initial Fe(III) concentration was 1.48 mM in NaOAc buffer, pH 3.6. A Fe(II) standard curve determined under the same conditions was used as a reference (●).

obtained for the ferric reduction by betalamic acid relative to the signal obtained for the calibration curve. Both slopes were calculated, and on the basis of the relation between these slopes, the number of electrons involved was determined to be two. Each betalamic acid molecule was able to directly reduce two Fe(III) ions to Fe(II).

For the first time, betalamic acid has been obtained and purified using a chromatographic method that yields sufficient material to characterize the properties of this compound. The results demonstrate that the extraordinary antiradical capacity of betalains is linked to its extended conjugate system. The results obtained in this study also explain the pH dependence of the antiradical capacity of betalamic acid. The pure structural unit of betalains might have applications in the food and pharmaceutical industries in its free form or as a starting material to obtain existing or new betalains.

EXPERIMENTAL SECTION

General Experimental Procedures. A Uvikon 940 spectrophotometer (Kontron Instruments, Zurich, Switzerland) attached to a Tectron thermostatic bath (JP Selecta, Barcelona, Spain) was used for absorbance spectroscopy. Measurements were taken in H_2O at 25 °C. The molar absorption coefficient at 536 nm, ε = 65 000 $M^{-1}\cdot cm^{-1}$, was used for the quantification of betanin.[35,36] The molar absorption coefficient for betalamic acid was determined using an end-point method by performing a set of betanin degradation experiments. The spectra of betanin solutions of known concentrations were recorded with the above instrument, and then the solutions were submitted to basic hydrolysis using ammonia at a final concentration of 1.2 M. The process was monitored spectrophotometrically for 30 min. Spectra were taken at 2 min intervals with a scan speed of 2000 nm·min^{-1}. The resulting betalamic acid solution corresponded to the initial pigment concentration, and the molar absorption coefficient at the wavelength corresponding to the absorbance maximum was calculated. Enzyme assays were also performed with the above-mentioned spectrophotometer using mushroom tyrosinase (EC 1.14.18.1), which was purchased from Sigma. The compound 4-methylcatecholquinone was prepared from 3.6 mM 4-methylcatechol with the addition of 130 units/mL tyrosinase. After the reaction ended, the quinones were filtered through Biomax-10 membranes (Millipore) to remove the catalyst. Filtered 4-methylcatecholquinone was used at a final concentration of 200 μM. Betalamic acid reaction assays were performed in 50 mM Na_3PO_4 buffer, pH 7.0. Other conditions are described in the text and figure legends. The reactions were followed by recording spectra at 1 min intervals with a scan speed of 2000 nm·min^{-1} or by measuring the absorbance at 530 nm. The lag period was calculated as the intercept on the abscissa obtained by extrapolation of the linear part of the product accumulation curve. No detectable evolution was detected in the absence of the quinone. Measurements were performed in triplicate, and the mean values and standard deviations were plotted. In each case, the errors associated with the results correspond to the residual standard deviations. Kinetic data analysis was performed by nonlinear regression fitting[44] using SigmaPlot Scientific Graphing for Windows version 8.0 (2001, SPSS Inc.). A Shimadzu LC-10A apparatus (Kyoto, Japan) equipped with a SPD-M10A photodiode array detector (PDA) was used for analytical HPLC separations. Reversed-phase chromatography was performed with a 250 × 4.6 mm Kromasil 100 C-18 column packed with 5 μm particles (Teknokroma, Barcelona, Spain). Gradients were formed with two He-degassed solvents. Solvent A was H_2O containing 0.05% TFA, and solvent B was MeCN containing 0.05% TFA. A linear gradient was performed for 25 min from 0% B to 35% B. The flow rate was 1 mL·min^{-1}, and the operating temperature was 25 °C. The injection volume was 20 μL. An Agilent VL 1100 apparatus with an LC/MSD trap (Agilent Technologies, Palo Alto, CA, USA) was used for the HPLC-ESIMS analysis. The elution conditions were as described above using a Zorbax SB-C18 (30 × 2.1 mm, 3.5 μm) column (Agilent Technologies) with a flow rate of 0.3 mL min^{-1}. The vaporizer temperature was 350 °C, and the voltage was maintained at 3.5 kV. The sheath gas was N_2 at 45 psi. Samples were ionized in the positive mode. The ion monitoring mode was full scan in the 50−600 m/z range. The electron multiplier voltage for detection was 1350 V. All chemicals and reagents were obtained from Sigma (St. Louis, MO, USA). HPLC-grade MeCN was purchased from Labscan Ltd. (Dublin, Ireland). Distilled H_2O was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Betanin was extracted and purified from red beet roots.[33]

Betalamic Acid Purification. Betalamic acid was purified by anionic exchange chromatography after betanin hydrolysis in an Äkta purifier apparatus (General Electric Healthcare, Milwaukee, WI, USA). The equipment was operated with a PC running Unikorn software version 3.00. The purification solvents used were 20 mM Na_3PO_4 buffer, pH 6.0 (solvent A), and the same buffer, containing 2 M NaCl (solvent B). Protocol A: A 25 × 7 mm, 1 mL Q-Sepharose Fast Flow column (cross-linked agarose with quaternary ammonium as the exchanger group, 90 μm particle size) was purchased from General Electric Healthcare. After sample injection, the elution process was as follows: 0% B from the beginning to 7.0 mL, followed by a linear gradient from 0% B to 35% B over 20 mL. One-milliliter fractions were collected. The injection volume was 100 μL of hydrolysis medium (at the hydrolysis pH), and the flow rate was 1.0 mL·min^{-1}. Protocol B: A 25 × 16 mm, 5 mL Q-Sepharose Fast Flow column (General Electric Healthcare) was used for scaled-up purification. The elution process was as follows: 0% B from the beginning to 65 mL, followed by a linear

gradient from 0% B to 35% B over 100 mL. The injection volume was 10 mL of hydrolysis medium. Two-milliliter fractions were collected, and the flow rate was 1.0 mL min^{-1}.

Free Radical Scavenging Activity. The antiradical capacity of betalamic acid was evaluated by following its effect on the stable free radical $ABTS^{\bullet+}$. The decolorization of the $ABTS^{\bullet+}$ solutions was monitored spectrophotometrically at λ = 734 nm.[15] The $ABTS^{\bullet+}$ radical was prepared using 2 mM ABTS solutions and peroxidase (88 units/L commercial horseradish peroxidase type VI, purchased from Sigma) in the presence of H_2O_2 (45 μM) in 12 mM NaOAc buffer, pH 5.0. The reaction was diluted by 2/3 by the addition of samples, and the reactions were performed in 53 mM Na_3PO_4 buffer, pH 7.0. Other conditions are as specified in the text. Measurements of the absorbances of the 96-well plates were performed after 24 h incubations at 20 °C in a Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT, USA). All measurements were performed in duplicate, and the mean values were plotted. The final volume in each well was 300 μL (calculated path length = 0.87 cm), and the sample volume added was 20 μL. Detector linearity under the assay conditions was confirmed (r = 0.9993).

Antioxidant Capacity. The antioxidant power of betalamic acid was characterized by the reduction of Fe(III) to Fe(II). The method used to evaluate FRAP was performed as described by Benzie and Strain.[43] Briefly, $FeCl_3$ solutions at a final concentration of 1.48 mM in 223 mM NaOAc buffer, pH 3.6, were used. The reduction of Fe(III) to Fe(II) was observed through the addition of the agent 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) at a final concentration of 741 μM; this compound forms a colored complex with Fe(II). The reduction reaction was followed spectrophotometrically at λ = 593 nm (Uvikon 940 spectrophotometer). All measurements were performed in duplicate, and the mean values and standard deviations were plotted.

AUTHOR INFORMATION

Corresponding Author

*Phone: +34 868 884786. Fax: +34 868 884147. E-mail: fgandia@um.es.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Ministerio de Ciencia e Innovación (MICINN, FEDER, Spain) (Projects AGL2010-17938 and AGL2011-25023) and by the Programa de Ayudas a Grupos de Excelencia de la Región de Murcia, de la Fundación Séneca, Agencia de Ciencia y Tecnología de la Región de Murcia (Plan Regional de Ciencia y Tecnología 2007/2010). F.G.-H. has a contract with the "Programa Ramón y Cajal" (MICINN, FEDER, Spain).

REFERENCES

(1) Moreno, D. A.; García-Viguera, C.; Gil, J. I.; Gil-Izquierdo, A. *Phytochem. Rev.* **2008**, *7*, 261−280.

(2) Musso, H. *Tetrahedron* **1979**, *35*, 2843−2853.

(3) von Ardenne, R.; Döpp, H.; Musso, H.; Steiglich, W. *Z. Naturforsch.* **1974**, *29c*, 637−639.

(4) Svenson, J.; Smallfield, B. M.; Joyce, N. I.; Sanson, C. E.; Perry, N. B. *J. Agric. Food Chem.* **2008**, *56*, 7730−7737.

(5) Wyler, H.; Mabry, T. J.; Dreiding, A. S. *Helv. Chim. Acta* **1963**, *46*, 1745−1748.

(6) Piattelli, M.; Minale, L.; Prota, G. *Tetrahedron* **1964**, *20*, 2325−2329.

(7) Piattelli, M. *The Biochemistry of Plants*; Conn, E. E., Ed.; Academic Press Inc.: New York, 1981; Vol. 7, pp 557−575.

(8) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. *Plant Physiol.* **2005**, *138*, 421−432.

(9) Gandía-Herrero, F.; García-Carmona, F.; Escribano, J. *J. Agric. Food Chem.* **2004**, *52*, 609−615.

(10) Sasaki, N.; Abe, Y.; Goda, Y.; Adachi, T.; Kasahara, K.; Ozeki, Y. *Plant Cell Physiol.* **2009**, *50*, 1012−1016.

(11) Piattelli, M.; Minale, L.; Prota, G. *Phytochemistry* **1965**, *4*, 121−125.

(12) Butera, D.; Tesoriere, L.; Di Gaudio, F.; Bongiorno, A.; Allegra, M.; Pintaudi, A. M.; Kohen, R.; Livrea, M. A. *J. Agric. Food Chem.* **2002**, *50*, 6895−6901.

(13) Gandía-Herrero, F.; Jiménez-Atiénzar, M.; Cabanes, J.; García-Carmona, F.; Escribano, J. *J. Agric. Food Chem.* **2010**, *58*, 10646−10652.

(14) Stintzing, F. C.; Schieber, A.; Carle, R. *Planta Med.* **1999**, *65*, 632−5.

(15) Escribano, J.; Pedreño, M. A.; García-Carmona, F.; Muñoz, R. *Phytochem. Anal.* **1998**, *9*, 124−127.

(16) Pavlov, A.; Kovatcheva, P.; Georgiev, V.; Koleva, I.; Ilieva, M. *Z. Naturforsch.* **2002**, *57c*, 640−644.

(17) Cai, Y.; Sun, M.; Corke, H. *J. Agric. Food Chem.* **2003**, *51*, 2288−2294.

(18) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. *J. Nat. Prod.* **2009**, *72*, 1142−1146.

(19) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. *Planta* **2010**, *232*, 449−460.

(20) Kapadia, G. J.; Azuine, M. A.; Sridhar, R.; Okuda, Y.; Tsuruta, A.; Ichiishi, E.; Mukainake, T.; Takasaki, M.; Konoshima, T.; Nishino, H.; Tokuda, H. *Pharmacol. Res.* **2003**, *47*, 141−148.

(21) Lu, X.; Wang, Y.; Zhang, Z. *Eur. J. Pharmacol.* **2009**, *615*, 223−227.

(22) Tesoriere, L.; Butera, D.; D'Arpa, D.; Di Gaudio, F.; Allegra, M.; Gentile, C.; Livrea, M. A. *Free Radical Res.* **2003**, *37*, 689−696.

(23) Tesoriere, L.; Butera, D.; Allegra, M.; Fazzari, M.; Livrea, M. A. *J. Agric. Food Chem.* **2005**, *53*, 1266−1270.

(24) Kujala, T. S.; Vienola, M. S.; Klika, K. D.; Loponen, J. M.; Pihlaja, K. *Eur. Food Res. Technol.* **2002**, *214*, 505−510.

(25) Wybraniec, S.; Platzner, I.; Geresh, S.; Gottlieb, H. E.; Haimberg, M.; Mogilnitzki, M.; Mizrahi, Y. *Phytochemistry* **2001**, *58*, 1209−1212.

(26) Cai, Y.; Sun, M.; Corke, H. *J. Agric. Food Chem.* **2001**, *49*, 1971−1978.

(27) Martínez-Parra, J.; Muñoz, R. *J. Agric. Food Chem.* **1997**, *45*, 2984−2988.

(28) Fincan, M.; De Vito, F.; Dejmek, P. *J. Food Eng.* **2004**, *64*, 381−388.

(29) Büchi, G.; Fliri, H.; Shapiro, R. *J. Org. Chem.* **1977**, *42*, 2192−2194.

(30) Wyler, H.; Wilcox, M. E.; Dreiding, A. S. *Helv. Chim. Acta* **1965**, *48*, 361−366.

(31) Huang, A. S.; von Elbe, J. H. *J. Food Sci.* **1985**, *50*, 1115−1120.

(32) Schliemann, W.; Kobayashi, N.; Strack, D. *Plant Physiol.* **1999**, *119*, 1217−1232.

(33) Gandía-Herrero, F.; García-Carmona, F.; Escribano, J. *Phytochem. Anal.* **2006**, *17*, 262−269.

(34) Escribano-Cebrián, J.; García-Carmona, F.; Gandía-Herrero, F. European Patent ES 2,349,522, 2009.

(35) Schwartz, S. J.; von Elbe, J. H. *J. Agric. Food Chem.* **1980**, *28*, 540−543.

(36) Trezzini, G. F.; Zrÿd, J.-P. *Phytochemistry* **1991**, *30*, 1901−1904.

(37) Kerem, Z.; Regev-Shoshani, G.; Flaishman, M. A.; Sivan, L. *J. Nat. Prod.* **2003**, *66*, 1270−1272.

(38) Valero, E.; Escribano, J.; García-Carmona, F. *Phytochemistry* **1988**, *27*, 2055−2061.

(39) García-Carmona, F.; Valero., E.; Cabanes, J. *Phytochemistry* **1988**, *27*, 1961−1964.

(40) Gliszczyńska-Świgło, A.; Szymusiak, H.; Malinowska, P. *Food Addit. Contam.* **2006**, *23*, 1079−1087.

(41) Madsen, H. L.; Andersen, C. M.; Jørgensen, L. V.; Skibsted, L. H. *Eur. Food Res. Technol.* **2000**, *211*, 240−246.

(42) Muzolf, M.; Szymusiak, H.; Gliszczyńska-Świgło, A.; Rietjens, I. M. C. M.; Tyrakowska, B. E. *J. Agric. Food Chem.* **2008**, *56*, 816−823.

(43) Benzie, I. F. F.; Strain, J. J. *Anal. Biochem.* **1996**, *239*, 70−76.

(44) Marquardt, D. W. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431−441.