Journal of Inorganic Biochemistry 104 (2010) 732–739

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

The chemical mechanism for Al^{3+} complexing with delphinidin: A model for the bluing of hydrangea sepals

Henry D. Schreiber *, Amy M. Swink, Taylor D. Godsey

Department of Chemistry, Virginia Military Institute, Lexington, VA 24450, United States

ARTICLE INFO

Article history:
Received 16 June 2009
Received in revised form 5 March 2010
Accepted 12 March 2010
Available online 20 March 2010

Keywords:
Aluminum
Anthocyanin
Bluing
Delphinidin
Hydrangea

ABSTRACT

The blooms of many hydrangea cultivars can be red or blue, with the color depending on the soil pH. This dependence reflects the availability of Al^{3+} to the plant under acidic conditions, as Al^{3+} changes the color of the anthocyanin pigment in hydrangea sepals from red to blue. A chemical model, Al^{3+} and delphinidin in acidic ethanol, was developed to understand the spectral characteristics and bluing of the hydrangea sepals. Delphinidin as its flavylium cation leads to red solutions in the model system. In the presence of Al^{3+}, the Al^{3+} removes H^+ ions from delphinidin, transforming delphinidin's flavylium cation to its blue quinoidal base anion which complexes with the Al^{3+}. To further stabilize this complex, a second flavylium cation stacks on top of the complexed quinoidal base anion, creating a bathochromic shift of the cation's spectral signature and accentuating the blue color. This Al^{3+}-delphinidin entity forms in adequate concentration for bluing only if there is a sufficient excess of Al^{3+}, the exact excess being a function of pH and concentration. The role of Al^{3+} in bluing is not just to form a primary complex with delphinidin, but also to create a template for the stacking of delphinidin (or possibly co-pigments).

© 2010 Elsevier Inc. All rights reserved.

1. Perspective

1.1. Red vs. blue hydrangea sepals

Hydrangeas are common household shrubs, renowned for their colorful and bountiful floral inflorescences. These inflorescences consist of relatively few fertile flowers surrounded by a large mass of sterile "flowers" called sepals (sepals being modified leaves) [1]. The sepal color for many hydrangeas is sensitive to soil pH, with red-pink sepals for hydrangea grown in basic soils and blue-purple sepals when grown in acidic soils [2,3]. Hydrangeas are unique with respect to their inflorescences being *in situ* color indicators of soil acidity or pH [4].

Hydrangeas are also unique in that their red and blue sepal colors are generated from the same anthocyanin [5]. The principal red pigment in hydrangea sepals is the anthocyanin, delphinidin-3-glucoside [5–9]. This anthocyanin can react with aluminum (as Al^{3+}) to form a blue complex [5,8–10], which may be intensified in color by the presence of co-pigments in the sepals [9–14]. As Al^{3+} is incorporated into hydrangea shrubs in acidic soils but not in basic soils, the soil pH (and, thus, the sepal color) is merely tracking the availability of aluminum [15].

Evidently, the hydrangea shrub has evolved into one of the few plants that can tolerate relatively high concentrations of Al^{3+} in the soil. In the presence of acidic soils, the root system of the hydrangea secretes citric acid into the soil [16]. The citric acid reacts with Al^{3+} in the soil to form a complex, which is then readily absorbed into the roots. In essence, this organic acid complex of Al^{3+} detoxifies aluminum for the shrub [17–19]. The Al^{3+} as the citric acid complex is then transported through the plant, to be eventually concentrated in the vacuoles of the sepals where it manifests itself as the blue Al^{3+} complex with the resident anthocyanin pigment. In basic soils, Al^{3+} is not available for plant uptake, probably precipitating in the soil as $Al(OH)_3$. Accordingly, the color of the anthocyanin remains red in these sepals.

1.2. The chemical mechanism for bluing

Others have previously studied and identified key features of the complex that causes the bluing in hydrangea sepals [5,8,20–23]. Fig. 1 shows the chemical structure of the red flavylium cation of delphinidin-3-glucoside, as well as the aluminum complex that transforms the delphinidin-3-glucoside to the blue quinoidal base anion. Others have also identified co-pigments, such as chlorogenic acid, that are integral to the stabilization of this complex [12,13]. However, bluing has been attributed principally to the blue anionic form of the quinoidal base of delphinidin-3-glucoside acting as a ligand for the Al^{3+} complex [5,20,24–26].

1.3. Objectives

The goals of this study are threefold: {1} to provide a spectral characterization of the Al^{3+}-anthocyanin complex that is central to

* Corresponding author. Tel.: +1 540 464 7416; fax: +1 540 464 7261.
E-mail address: HS@vmi.edu (H.D. Schreiber).

0162-0134/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2010.03.006

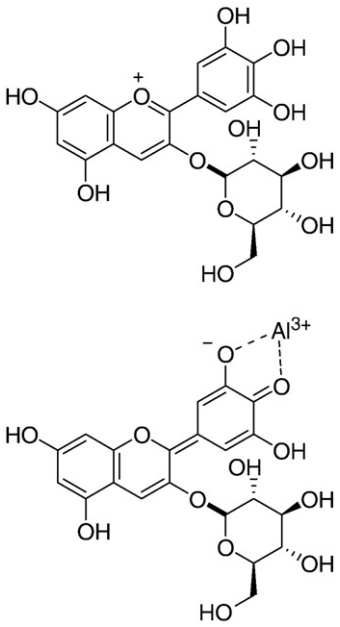

Fig. 1. Delphinidin-3-glucoside as its red flavylium cation form (top) and as one resonance structure of its blue quinoidal base anionic form complexed with Al^{3+} (bottom).

bluing in a model chemical system, {2} to develop a molecular mechanism for the formation and stabilization of the complex in the model system, and {3} to apply this understanding to explain certain aspects of the bluing of hydrangea sepals in the presence of Al^{3+}. The impact of metal ions in initiating color changes in floral pigments is greatly underappreciated [27–29]. Understanding the fundamentals of this bluing chemistry in hydrangea sepals may allow the design of novel chromophores (for example, ones that may produce yellow or orange sepals in hydrangea) in floral pigments using metal ions other than Al^{3+}.

2. Experimental

2.1. Model chemical system

Although delphinidin-3-glucoside is the primary pigment in hydrangea sepals, this study employed the core delphinidin molecule as the model colorant. As only the delphinidin portion of the pigment is involved in complexation with Al^{3+}, the attached sugar was assumed not to contribute significantly to the basic chemical mechanism for any color change [24,26,31–33]. Fig. 2 shows the structure of the red flavylium cation ($D^{+}_{f(red)}$) of delphinidin. Transformation to the blue quinoidal base anion ($D^{-}_{q(blue)}$) of delphinidin, also shown in Fig. 2, is pH dependent and requires the transfer of two hydrogen ions (H^{+}).

The solvent system is acidified ethanol, defined as 0.04 vol.% HCl in absolute ethanol. The acid stabilized delphinidin's red flavylium cation in the model system, much like the default coloration in hydrangea sepals is red. Use of ethanol as the solvent instead of an aqueous buffer further stabilized the flavylium cation, eliminating possible competing hydrolysis reactions that would eventually lead to near-colorless chalcones [33–36].

This model organic-based system was developed to characterize and understand the fundamental bluing reaction, not to replicate the bluing in the complex chemical medley of the natural sepal. Although the chemical mechanism for bluing was assumed to be analogous in both the simple and complex systems, some characteristics of the Al^{3+}-delphinidin complex in this model system may not be the same as in aqueous solutions. Nevertheless, substitution of just delphinidin for

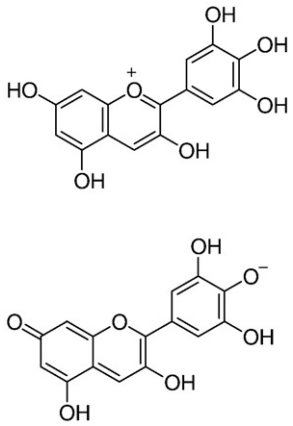

Fig. 2. Delphinidin, as the core portion of delphinidin-3-glucoside, in its flavylium cationic form (top) and one resonance form of its quinoidal base anion (bottom).

delphinidin-3-glucoside and of acidic ethanol for an aqueous buffer in this model has allowed the focus to be on color changes resulting from core anthocyanidin interactions with Al^{3+}.

2.2. Chemicals and Instrumentation

Aluminum chloride ($AlCl_3{\cdot}6H_2O$), hydrochloric acid (HCl), and absolute ethanol (C_2H_5OH) were reagent grade and used as received. Research-grade delphinidin chloride was used as received from Indofine Chemical Company. Solutions were prepared by standard gravimetric and volumetric procedures. Spectra were measured on a Shimadzu 3100 UV/vis/NIR spectrophotometer operating in the visible region with acidified ethanol as the reference.

2.3. Delphinidin in acidified ethanol

The visible spectrum of delphinidin in acidified ethanol is shown in Fig. 3. Delphinidin solutions have an intense red coloration even at micro-molar concentrations. The spectrum is characterized by an asymmetric absorption peak with a maximum at 559 nm. The absorbing species is delphinidin as its red flavylium cation.

Fig. 3 also shows that the absorbance of the flavylium cation of delphinidin obeys Beer's Law over a wide concentration range. The experimental molar absorptivity of 33,100 L mol^{-1} cm^{-1} is in general agreement with prior studies [37].

3. Results

3.1. Nature of the primary Al^{3+}–delphinidin complex

Upon adding Al^{3+} to delphinidin solution in acidified ethanol, an absorbance attributed to the Al^{3+}–delphinidin complex arises at about 616 nm. The solution's spectrum is exemplified in Fig. 4 for equimolar concentrations (19 μM) of Al^{3+} and delphinidin. The spectrum of the complex is resolved by subtracting the appropriately scaled flavylium cation spectrum (as shown in Fig. 3) from the Al^{3+}–delphinidin spectrum. As identified in prior studies [26,38], the complex's characteristic absorbance at 616 nm is attributed to delphinidin as its blue quinoidal base anion. Thus, the 616 nm peak is caused not by the complex with Al^{3+} *per se*, but by the change in structural form of the delphinidin. Fig. 4 illustrates that the peak at 616 nm only contributed less than 10% of the total solution's absorbance, one still dominated by the flavylium cation at these concentrations. Accordingly, the solution characterized in Fig. 4 is still red.

Job's Method (method of continuous variation) was used to study the stoichiometry of the Al^{3+}–delphinidin complex. The resolved

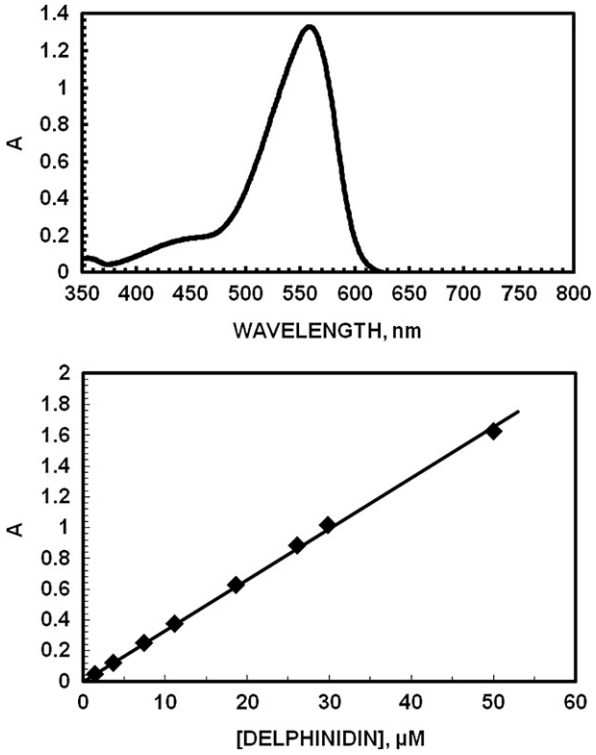

Fig. 3. The visible spectrum of 38 μM delphinidin, as its flavylium cation, in acidic ethanol (top). Beer's Law relationship of the delphinidin absorbance peak at 559 nm (bottom).

"complex" absorbance at 616 nm was monitored as a function of the relative molar concentration of delphinidin (with respect to Al^{3+}), as shown in Fig. 5. The plot indicates a 1:1 molar stoichiometric complex of delphinidin and Al^{3+}, which is in agreement with previous studies of this complex and of complexes of Al^{3+} with analogous molecules [5,20,21,26,38].

The "complex" absorbance at 616 nm is representative of the quinoidal base anion of delphinidin. In order for delphinidin to change from the flavylium cation to the quinoidal base anion, the delphinidin must lose two hydrogen ions. Thus, the role of Al^{3+} in the formation of the primary Al^{3+}–delphinidin complex must be two-fold: first to extract H^+ ions from the flavylium cation $D^+_{f(red)}$ so that delphinidin is converted to its quinoidal base anion $D^-_{q(blue)}$, and second to simultaneously stabilize

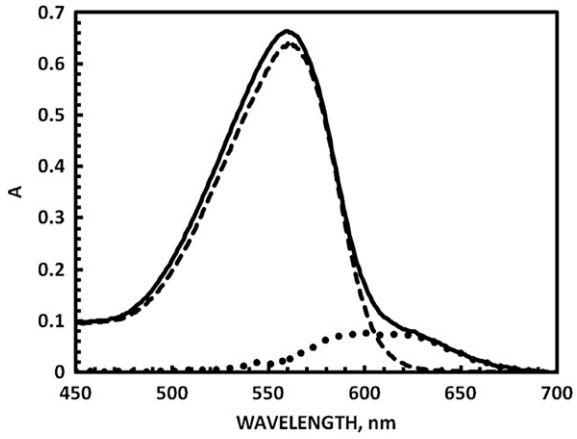

Fig. 4. Resolution of the visible spectrum (solid line) of a solution of 19 μM delphinidin and 19 μM Al^{3+} in acidic ethanol. Underlying components are attributed to the flavylium cation (dashed line) and quinoidal base anion, or complexed, (dotted line) forms of delphinidin.

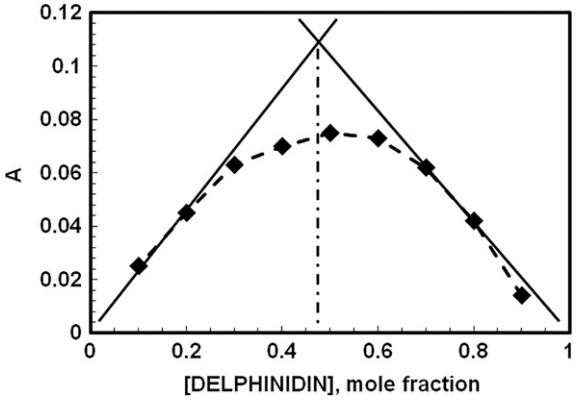

Fig. 5. Job's Method analysis of the Al^{3+}-delphinidin complex. The absorbance of the 616 nm peak (due to complexed delphinidin) is plotted as a function of the delphinidin mole fraction. Delphinidin mole fraction is defined as the moles of delphinidin divided by the total moles of delphinidin and Al^{3+}. Equimolar (38 μM) solutions of delphinidin and Al^{3+} were mixed in varying proportions in acidic ethanol.

the quinoidal base anion in the $[D_{q(blue)}AlCl_4]^{2-}$ complex. This process is illustrated by the following chemical equation:

$$D^+_{f(red)} + [AlCl_6]^{3-} \rightleftharpoons [D_{q(blue)}AlCl_4]^{2-} + 2H^+ + 2Cl^- \quad (1)$$

This equation assumes that Al^{3+} exists initially as a chloro-complex in the acidified ethanol; but the assumption is not critical for this study. It is likely that the ethanol (as the solvent) also contributes to the solvation sphere of Al^{3+}. The chloro-complex is used for simplicity in Eq. (1).

At a total concentration range of about 30 to 50 μM (for delphinidin and Al^{3+}), the complex peak at 616 nm is always small with respect to the entire spectral signature. The system remains red; evidently, the equilibrium constant for Eq. (1) is such that significant amounts of the blue quinoidal base anion (and, consequently, the complex) are not formed at this concentration.

3.2. The bluing of delphinidin by excess aluminum

The primary experimental strategy to form higher concentrations of the blue complex (that is, shift Eq. (1) to the products) was to increase the concentration of Al^{3+} (according to Le Châtelier's principle) from that used in the Job's Method study. In essence, a solution of 6200 μM Al^{3+} was added in varying amounts to delphinidin solutions, keeping delphinidin concentration approximately constant at 30–38 μM (and in a second study, delphinidin was kept constant at ≈16 μM). As Al^{3+} was systematically added to the delphinidin solution in ever increasing concentrations, the initially red solution turned purple, then eventually blue. A factor of thirty molar excess of Al^{3+} over delphinidin was required in this system to generate a visually pure blue, an excess that was in general agreement with other studies [9,39].

The spectral features of the Al^{3+}–delphinidin system in acidic ethanol are shown in Fig. 6, which consists of a series of three spectra of varying Al^{3+} concentration but constant delphinidin concentration. Two aspects of the spectra are immediately obvious with increasing Al^{3+} concentration: first the flavylium cation absorbance systematically shifts from 559 nm to 589 nm as it becomes less of a contributor to the total spectrum, and second the complex or quinoidal base anion component remains at 616 nm but increases in absorbance. The two contributions to the measured spectrum were resolved by successive approximations. The absorbance and shape of the flavylium cation spectrum was scaled (in absorbance) and shifted (in wavelength) such that when it was subtracted from the experimental spectrum, a symmetric quinoidal base anion spectrum was generated. In a sense, the flavylium cation peak is kept the same in terms of asymmetric

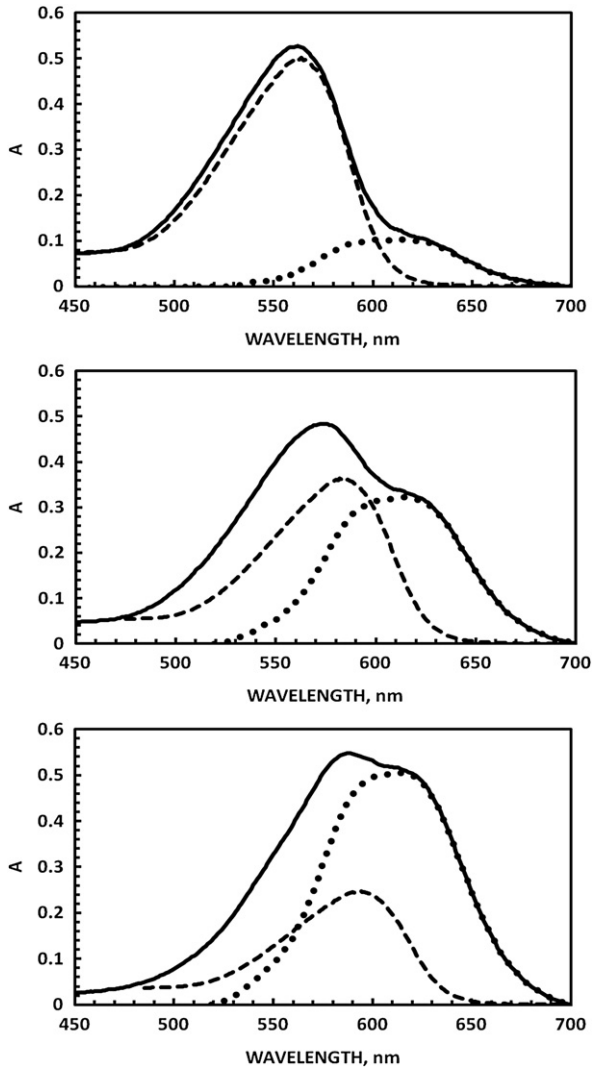

Fig. 6. Visible spectra of solutions of delphinidin and Al^{3+} in acidic ethanol. 16 μM delphinidin and 60 μM Al^{3+} (top), 16 μM delphinidin and 200 μM Al^{3+} (middle), and 16 μM delphinidin and 480 μM Al^{3+} (bottom) in acidic ethanol. Spectra (solid lines) were resolved into flavylium cation (dashed lines) and quinoidal base anion (dotted lines) contributions.

spectral shape and Beer's Law but allowed to vary in terms of wavelength of maximum absorbance.

In order to ascertain whether the assumption that the shape-absorbance characteristics of the flavylium cation remain constant despite its wavelength shift, the concentration of the complexed delphinidin (quinoidal base anion) was calculated by the following equation:

$$[\mathrm{Al}\text{–}\mathrm{D}^{-}_{\mathrm{q(blue)}}] = [\Sigma\mathrm{D}]\text{–}[\mathrm{D}^{+}_{\mathrm{f(red)}}] \quad (2)$$

that is, by subtracting the flavylium cation concentration (determined from its peak absorbance divided by its molar absorptivity, previously determined to be 33,100 L mol^{-1} cm^{-1}) from the initial or total concentration of delphinidin. In other words, delphinidin must occur in either of its two forms, with the quinoidal base anionic form complexed with Al^{3+}. Consequently, Fig. 7 shows the Beer's Law plot for the complex peak at 616 nm. The linearity of the plot as well as the reasonable molar absorptivity of 61,600 L mol^{-1} cm^{-1} for the quinoidal base anionic form of delphinidin in the complex provide credence to the basic assumptions of the spectral interpretation. Subsequently, the percent of delphinidin complexed with Al^{3+}, or in

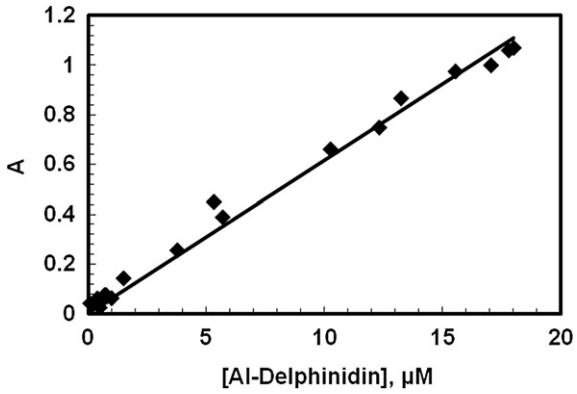

Fig. 7. Beer's Law plot for the resolved 616 nm absorbance peak attributed to the quinoidal base anionic form of delphinidin complexed with Al^{3+}.

essence the percent of delphinidin existing as the quinoidal base anion, could then be calculated for each solution.

Fig. 8 then illustrates the two underlying and simultaneous causes for the bluing of delphinidin solutions by Al^{3+}. The percent of complexed delphinidin (blue quinoidal base anion) steadily increases with increasing relative Al^{3+} concentration until the complexed delphinidin plateaus at about 50% of all delphinidin, once the Al^{3+} is present in at least thirty-fold molar excess. Concurrent with this effect is the bathochromic shifting of the flavylium cation peak by 30 nm (559 to 589 nm). With increasing Al^{3+} concentration, the "red" flavylium cation, in effect, becomes the "blue" flavylium cation.

In order to further investigate the bluing mechanism, the effect of absolute concentration of both delphinidin and Al^{3+} on the bluing was studied. Fig. 9 shows spectra of four samples at a constant Al^{3+}: delphinidin mole ratio of 50:1. At a concentration ratio of 50 μM to 1 μM, the solution is pink; whereas at 600 μM to 12 μM, it is blue. At the intermediate concentrations, the solutions systematically change from purple to blue. The spectra once again demonstrate the steadily increasing proportion (albeit eventually hitting a plateau) of the blue quinoidal base anionic complex at 616 nm, while the flavylium cation component's absorbance shifts to higher wavelength. Such spectral characteristics confirm the two underlying features of the delphinidin bluing by Al^{3+}. Analogous concentration effects have been used to

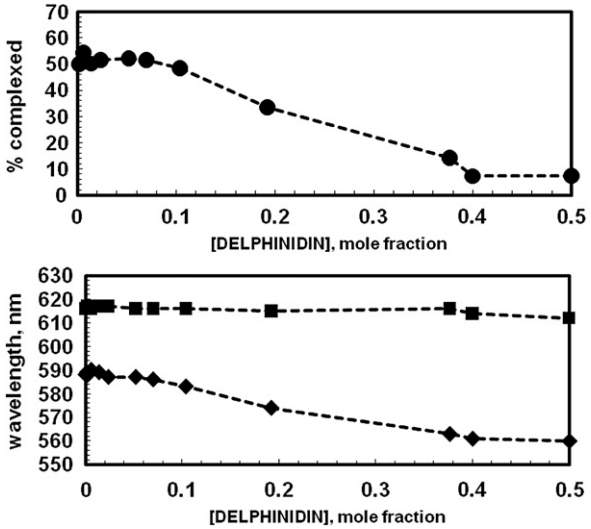

Fig. 8. % complexed delphinidin, or % as quinoidal base anion, (top) and wavelength of absorbance maximum for the flavylium cation as diamonds and for the blue quinoidal base anion as squares (bottom) as a function of the delphinidin mole fraction. Delphinidin mole fraction is defined in the caption for Fig. 5. Delphinidin concentration is approximately constant at 25–38 μM.

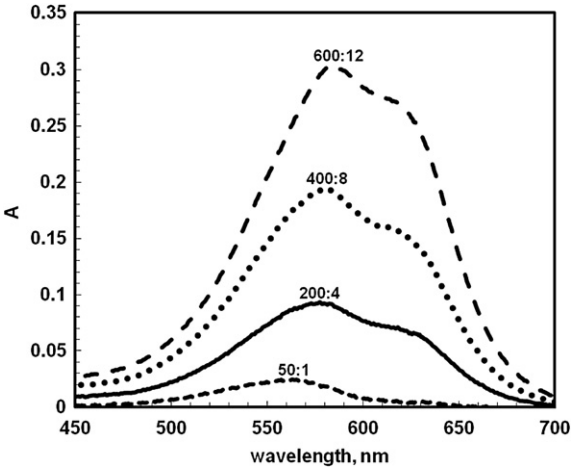

Fig. 9. Visible spectra of solutions of Al^{3+} and delphinidin with a constant mole ratio of 50:1 (Al^{3+}:delphinidin) in acidic ethanol. Actual μM concentrations of 50:1 (short-dashed line), 200:4 (solid line), 400:8 (dotted line), and 600:12 (long-dashed line) label each spectrum.

confirm self-association or π–π stacking of anthocyanidin components in complexes [40–42]. This conclusion is also consistent with a limit of 50% of all delphinidin forming the quinoidial base anionic complex with Al^{3+}; evidently the other half remains as the flavylium cation stacked on top of this primary complex.

We propose to represent the chemical bluing mechanism for the interaction of Al^{3+} with delphinidin by the following set of chemical equations, incorporating Eq. (1) as one part in the final complex formation:

$$D^{+}_{f(red)} + [AlCl_6]^{3-} \rightleftharpoons [D_{q(blue)}AlCl_4]^{2-} + 2H^{+} + 2Cl^{-} \quad (1)$$

$$D^{+}_{f(red)} + [D_{q(blue)}AlCl_4]^{2-} \rightleftharpoons [D^{+}_{f(blue)}][D_{q(blue)}AlCl_4]^{2-} \quad (3)$$

The first or primary step is the formation of the stable complex of Al^{3+} and delphinidin. The presence of Al^{3+} is the driving force for delphinidin to lose hydrogen ions and transform from its red flavylium cation ($D^{+}_{f(red)}$) to its blue quinoidal base anion ($D^{-}_{q(blue)}$). The Al^{3+} also establishes the template to stabilize this quinoidal base anion in a complex ion ($[D_{q(blue)}AlCl_4]^{2-}$). In the second and simultaneous step, the complex associates with another red flavylium cation of delphinidin by π–π stacking, accentuated by charge transfer interaction [43], to complete the complex. The associated flavylium cation ($D^{+}_{f(blue)}$) has similar spectral characteristics as the free flavylium cation, with the exception of the bathochromic shift in its spectrum, which enhances the red to blue color change with the addition of Al^{3+}. Other metal-based floral chromophores likewise employ the stacking of aromatic pigment constituents to generate color [5,29,30,44,45], in particular the stacking of anthocyanin units [46]. The Al^{3+}-delphinidin interaction employs both complex formation (Eq. (1)) and stacking (Eq. (3)) for its color change.

3.3. Effect of solvent pH

Increasing the pH of the solvent enhances the bluing effects of delphinidin by Al^{3+}. The quinoidal base anion of delphinidin is stabilized with respect to its flavylium cation form with increasing pH in aqeuous systems [24–26]. In addition, Eq. (1) shows the equilibrium should shift to form additional blue product with increasing pH (or decreasing H^{+} concentration) according to Le Châtelier's principle.

The pH of acidified ethanol (0.04 vol.% of HCl) was measured to be 0.9. Another solution of ethanol was also prepared to have a pH of 2.72 by mixing a few drops of HCl with sufficient ethanol. The pH of pure ethanol was measured to be about 8.

Fig. 10 shows the visible spectra of delphinidin in these three ethanolic solutions. All solutions had a red color (although a slight purplish tint in pure ethanol), with the spectral signature characteristic of delphinidin as its flavylium cation. The wavelength of the absorbance maximum was 559 nm, independent of ethanol pH. However, the intensity of the absorbance decreased with increasing ethanol pH; the molar absorptivity of the flavylium cation decreased from 33,100 L mol^{-1} cm^{-1} in the acidified ethanol, to 23,000 L mol^{-1} cm^{-1} in the ethanol with pH 2.72, and to 11,200 L mol^{-1} cm^{-1} in ethanol. Beer's Law behavior of the spectral absorbance in all solvents indicated the presence of delphinidin entirely in its flavylium cationic form.

The Al^{3+}-delphinidin solutions became blue at less of a molar Al^{3+} excess in slightly acidified ethanol (pH 2.72) and absolute ethanol than was observed for acidified ethanol. Spectra were resolved in a similar fashion for these solutions as done for acidified ethanol previously. Once again, the flavylium cation peak shifts from 559 nm to 590 nm (bathochromic shift of 31 nm), and the complex peak steadily grows at about 610 nm. Fig. 11 compares the spectra of ethanolic solutions containing about 62 μM Al^{3+} and 36 μM delphinidin at the three acidities. Whereas the solution is purple in acidified ethanol, the solutions are blue at the higher pHs. The blue complex peak dominates at the higher pH, but still never totally eliminates the flavylium cation peak.

Analogous to Fig. 7, Fig. 12 provides Beer's Law plots of the complex peak (610–616 nm) for all ethanolic solutions. Evidently, delphinidin occurs in the flavylium cation (free and/or associated) and quinoidal base anion (complexed) forms irrespective of the ethanolic pH. Like the molar absorptivity of the flavylium cation peak, the molar absorptivity of the quinoidal base anion decreases near-identically with increasing solvent pH; the molar absorptivity of the quinoidal base anion (or complexed delphinidin) changes from 61,600 L mol^{-1} cm^{-1} in the acidified ethanol, to 42,300 L mol^{-1} cm^{-1} in the ethanol with pH 2.72, and to 14,700 L mol^{-1} cm^{-1} in ethanol. The solvent must play an analogous role in the spectral characteristics of both types of delphinidin absorbances.

Fig. 13 compares the percent complexed delphinidin and spectral maxima as a function of the relative delphinidin concentration (with respect to Al^{3+} concentration) for the three ethanol solvents of varying pH. The percent complexed delphinidin always plateaus at about 50–60% in excess Al^{3+}, independent of ethanol pH; however, less of a molar Al^{3+} excess is required to achieve the plateau as the pH increases. In addition, less of a molar Al^{3+} excess is required to

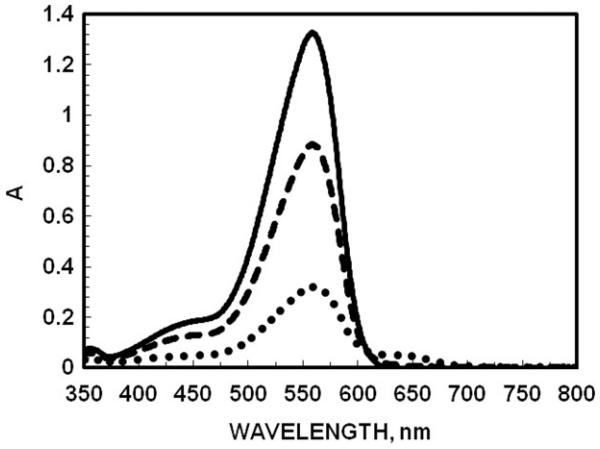

Fig. 10. The visible spectrum of 38 μM delphinidin, as its flavylium cation, in acidic ethanol (solid line), in ethanol with pH 2.72 (dashed line), and in pure ethanol (dotted line).

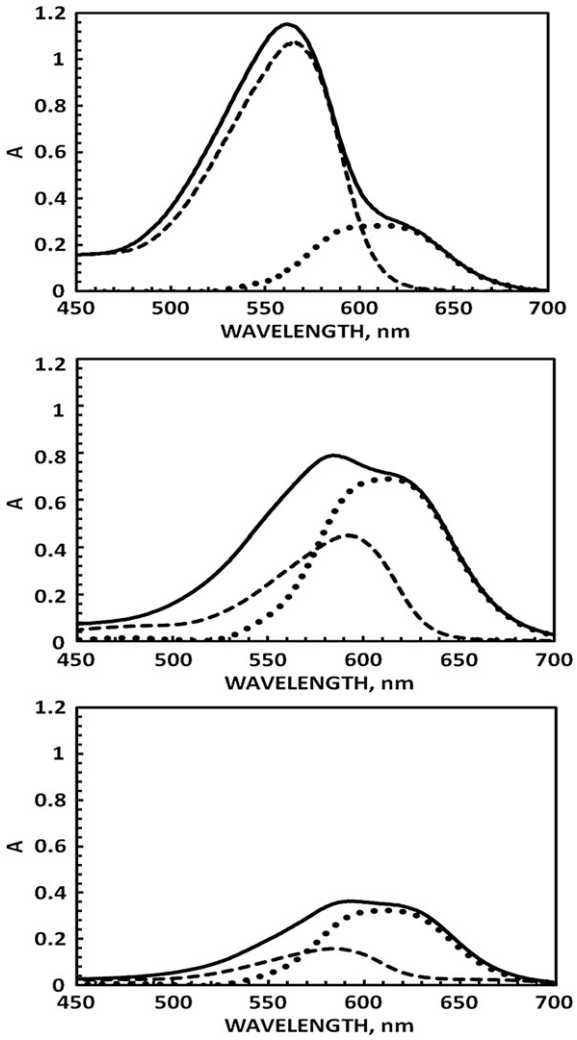

Fig. 11. Visible spectra of solutions of 35–38 μM delphinidin and 62 μM Al^{3+} in acidified ethanol (top), in slightly acidified ethanol of pH 2.72 (middle), and in ethanol (bottom). Spectra (solid lines) were resolved into flavylium cation (dashed lines) and quinoidal base anion (dotted lines) contributions.

achieve the full bathochromic shift of the flavylium cation as the pH increases. This observation is consistent with Eqs. (1) and (3), the mechanism in which the flavylium cation of delphinidin associates with the quinoidal base anionic complex as it forms.

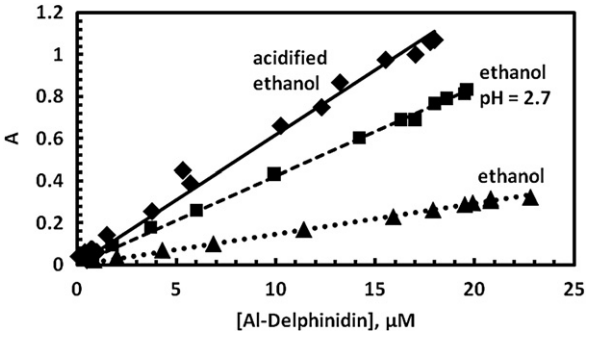

Fig. 12. Beer's Law plot for the resolved 616 nm absorbance peak attributed to the quinoidal base anionic form of delphinidin complexed with Al^{3+} in acidified ethanol (diamonds and solid line), in slightly acidified ethanol of pH 2.72 (squares and dashed line), and in ethanol (triangles and dotted line).

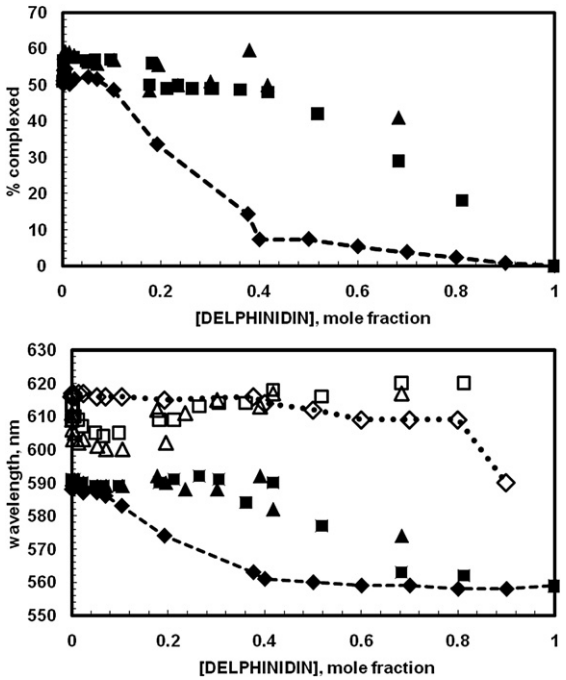

Fig. 13. % complexed delphinidin, or % as quinoidal base anion, (top) and wavelength of absorbance maximum for the flavylium cation as solid symbols and for the quinoidal base anion as open symbols (bottom) as a function of the delphinidin mole fraction and as a function of the solvent pH. Delphinidin mole fraction is defined in the caption for Fig. 5. Delphinidin concentration is approximately constant at 25–38 μM. Top graph: acidic ethanol (diamonds and dashed line), ethanol with pH of 2.72 (squares), and ethanol (triangles). Bottom graph: flavylium cation in acidic ethanol (solid diamonds and dashed line), ethanol with pH of 2.72 (solid squares), and ethanol (solid triangles); quinoidal base anion in acidic ethanol (open diamonds and dotted line), ethanol with pH of 2.72 (open squares), and ethanol (open triangles).

4. Discussion

4.1. The chemical model for bluing — the role of Al^{3+}

Aluminum as Al^{3+} complexing with delphinidin-3-glucoside is well-known to be the root cause of bluing in hydrangea sepals [5,8–10,23,47]. That is, the normally red flavylium cation of delphinidin-3-glucoside transforms to the blue quinoidal base anionic form upon complexation as shown in Eq. (1). However, this study describes an additional chemical intricacy of the bluing mechanism. Concurrent to the complexation, a second step of stacking or self-association occurs, enhancing the bluing. The bathochromic shift of the flavylium cation associating with the complex is a crucial part of the bluing mechanism as shown in Eq. (3).

The role of Al^{3+} in the bluing, then, is to initiate the loss of the hydrogen ions by the delphinidin to transform the flavylium cation to the quinoidal base anion, then to complex with the resulting quinoidal base anion. Simultaneously, this Al^{3+}-complex forms a template for the stacked association with a second flavylium cation entity. A pictorial representation of this blue complex, the product in Eq. (3), is shown in Fig. 14.

The exact coloration of the Al^{3+}–delphinidin system is controlled by multiple variables. The intensity of the coloration, whether red or blue, increases as the pH decreases (becomes more acidic). On the other hand, bluing occurs with less of an excess of Al^{3+} as the pH increases (becomes less acidic). In addition, both the intensity and the coloration can be controlled by not only the molar ratio of Al^{3+} to delphinidin, but also the absolute concentrations of the two components.

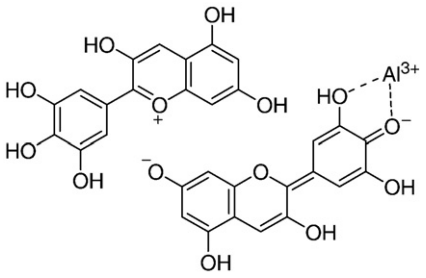

Fig. 14. Pictorial representation of the Al^{3+}-delphinidin complex in acidic ethanol. A quinoidal base anion of delphinidin forms a primary complex with Al^{3+}, and further a flavylium cation of delphinidin associates (by charge transfer and π–π interaction) with the quinoidal base anion. The resonance form of the quinoidal base anion is chosen to emphasize its electronic interaction with the flavylium cation. The flavylium cation stacks on top of the quinoidal base, not next to the quinoidal base anion as shown in this two-dimensional representation.

4.2. Application of mechanism to explain aspects of hydrangea bluing

This study emphasizes the key role that aluminum plays in the bluing of hydrangea sepals insofar as the chemical model approximates the natural system. Al^{3+} forms a blue complex with the normally red delphinidin-3-glucoside pigment. Bluing only occurs when Al^{3+} is in excess of the delphinidin pigment, but exact excesses depend on variables such as pH and concentration.

Some hydrangea cultivars have sepals that are known to be easy to blue, while others have sepals that are harder to blue [1]. Such cultivars have been categorized by their delphinidin-3-glucoside content [47]. In part, the ease of bluing may be related to the relative pigment content; that is, it takes more Al^{3+} to blue the sepals of the cultivars with higher delphinidin-3-glucoside contents. The chemical model with its need for a molar excess of Al^{3+} over the pigment gives credence to the fact that not all cultivars are equal in their bluing ability.

Another component in the bluing of hydrangea sepals is the presence of co-pigments. Co-pigments have been identified as necessary in the stabilization of the Al^{3+}-delphinidin complex [5,11–13]. By application of the chemical mechanism for bluing proposed in this study, the role of the co-pigment in the blue complex may be much like the stacked flavylium cation. In the sepal where a mix of delphinidin-3-glucoside, Al^{3+}, and co-pigments all co-exist — the co-pigment(s) may replace all or some of the delphinidin species stacked on top of the $[Al–D^{-}_{q(blue)}]$ complex, accentuating the bluing as well as stabilizing the complex. Similarly, Fe^{2+} stabilized an anthocyanin complex with a co-pigment stacked on top of the primary complex [48]. It remains to be identified whether a stable complex can be formed in the vacuolar part of the hydrangea sepal with just a stacked co-pigment and delphinidin-3-glucoside as its quinoidal base anion (and the absence of Al^{3+}, or the substitution of some other metal ion for Al^{3+}).

The bluing mechanism is just one part of the uniqueness of the hydrangea shrub to change sepal color with soil pH, or rather Al^{3+} availability. A second part is the ability of the shrub to incorporate and detoxify Al^{3+}. Al^{3+} is absorbed through the roots and transported throughout the shrub as a citrate complex. Accordingly, the formation of the stable Al^{3+} complex with the pigment in the sepals requires the exchange of ligands with the citrate complex in the cellular soup containing other potential ligands such as sugars, protein, and phosphates.

5. Conclusion

The fundamental chemical mechanism for the bluing of delphinidin by Al^{3+} in acidic ethanol can be represented by a two-step process. First, Al^{3+} extracts H^{+} ions from the normally red delphinidin pigment as its flavylium cation to produce the blue quinoidal base anion of delphinidin that complexes with the Al^{3+}. Second, this complex is stabilized by the π–π stacking of a second delphinidin as a flavylium cation on top of the $[Al^{3+}–D^{-}_{q(blue)}]$ complex. The stacking interaction accentuates the bluing through a bathochromic shift in spectral absorbance. According to this model, an analogous blue complex is probably the source of the bluing of hydrangea sepals initiated by the incorporation of Al^{3+} into the sepal.

6. Abbreviations

$D^{+}_{f(red)}$ delphinidin as the red flavylium cation
$D^{-}_{q(blue)}$ delphinidin as the blue quinoidal base anion
$[Al–D^{-}_{q(blue)}] = [D_{q(blue)}AlCl_4]^{2-}$ blue complex formed by Al^{3+} and the quinoidal base anion of delphinidin
$D^{+}_{f(blue)}$ delphinidin as the blue flavylium cation (stacked on the primary complex)

Acknowledgement

This research was supported by grants from the Thomas F. and Kate Miller Jeffress Memorial Trust and from the Virginia Military Institute.

References

[1] M.A. Dirr, Hydrangeas for American Gardens, Timber Press, Portland OR, 2004.
[2] K.K. Karukstis, G.R. Van Hecke, Chemical Connections: The Chemical Basis of Everyday Life, Academic Press, San Diego, 2000, pp. 63–66.
[3] R.C. Allen, Contrib. Boyce Thompson Inst. 13 (1943) 221–242.
[4] E.M. Chenery, Ann. Bot. 12 (1948) 121–136.
[5] K. Yoshida, M. Mori, T. Kondo, Nat. Prod. Rep. 26 (2009) 884–915.
[6] S. Asen, H.W. Siegelman, N.W. Stuart, Proc. Amer. Soc. Hort. Sci. 69 (1957) 561–569.
[7] K. Takeda, M. Kariuda, H. Itoi, Phytochemistry 24 (1985) 2251–2254.
[8] K. Yoshida, Y. Toyama-Kato, K. Kameda, T. Kondo, Plant Cell Physiol. 44 (2003) 262–268.
[9] D. Ito, Y. Shinkai, Y. Kato, T. Kondo, K. Yoshida, Biosci. Biotechnol. Biochem. 73 (2009) 1054–1059.
[10] Y. Toyama-Kato, K. Yoshida, E. Fujimori, H. Haraguchi, Y. Shimizu, T. Kondo, Biochem. Eng. J. 14 (2003) 237–241.
[11] K. Takeda, R. Kubota, C. Yagioka, Phytochemistry 24 (1985) 1207–1209.
[12] T. Kondo, Y. Toyama-Kato, K. Yoshida, Tetrahed. Lett. 46 (2005) 6645–6649.
[13] Y. Toyama-Kato, T. Kondo, K. Yoshida, Heterocycles 72 (2007) 239–254.
[14] K. Yoshida, D. Ito, Y. Shinkai, T. Kondo, Phytochemistry 69 (2008) 3159–3165.
[15] J.F. Ma, P.R. Ryan, E. Delhaize, Trends Plant Sci. 6 (2001) 273–278.
[16] J.F. Ma, S. Hiradate, K. Nomoto, T. Iwashita, H. Matsumoto, Plant Physiol. 113 (1997) 1033–1039.
[17] M. Osaki, T. Watanabe, T. Tadano, Soil Sci. Plant Nutrit. 43 (1997) 551–563.
[18] J.F. Ma, Plant Cell Physiol. 41 (2000) 383–390.
[19] J.F. Ma, J. Furukawa, J. Inorg. Biochem. 97 (2003) 46–51.
[20] O. Dangles, M. Elhabiri, R. Brouillard, J. Chem, Soc. Perkin Trans. 2 (1994) 2587–2596.
[21] M. Elhabiri, P. Figueiredo, K. Toki, N. Saito, R. Brouillard, J. Chem. Soc. Perkin Trans. 2 (1997) 355–361.
[22] D.S. Veselinovic, J.M. Baranac, Z.D. Zujovic, D.S. Djordjevic, J. Agric. Food Chem. 40 (1992) 2337–2340.
[23] K. Takeda, T. Yamashita, A. Takahashi, C. Timberlake, Phytochemistry 29 (1990) 1089–1091.
[24] G. Mazza, R. Brouillard, J. Agric. Food Chem. 35 (1987) 422–426.
[25] T.V. Mistry, Y. Cai, T.H. Lilley, E. Haslam, J. Chem. Soc. Perkin Trans. 2 (1991) 1287–1296.
[26] M.C. Moncada, S. Moura, M.J. Melo, A. Roque, C. Lodeiro, F. Pina, Inorg. Chim. Acta 356 (2003) 51–61.
[27] T. Goto, H. Tamura, T. Kawai, T. Hoshino, N. Harada, T. Kondo, Ann. NY Acad. Sci. 471 (1986) 155–173.
[28] T. Honda, N. Saito, Heterocycles 56 (2002) 633–692.
[29] G.A. Ellestad, Chirality 18 (2005) 134–144.
[30] K. Takeda, Proc. Jpn. Acad., Ser. B 82 (2006) 142–154.
[31] C.F. Timberlake, P. Bridle, J. Sci. Food Agric. 18 (1967) 473–485.
[32] C.E. Lewis, J.R.L. Walker, J.E. Lancaster, Food Chem. 54 (1995) 315–319.
[33] K. Torskangerpoll, O.M. Anderson, Food Chem. 89 (2005) 427–440.
[34] L. Jurd, J. Org. Chem. 28 (1963) 987–991.

[35] R. Brouillard, J.-E. Dubois, J. Amer. Chem. Soc. 99 (1977) 1359–1364.
[36] G.A. Iacobucci, J.G. Sweeny, Tetrahedron 39 (1983) 3005–3038.
[37] M.M. Giusti, R.E. Wrolstad, in: R.E. Wrolstad (Ed.), Current Protocols in Food Analytical Chemistry, John Wiley & Sons, New York, 2001, pp. F1.2.1–F1.2.13.
[38] J.M. Dimitric Markovic, Z.S. Markovic, J.M. Baranac, M.L. Dasic, Monatsh. fur Chemie 138 (2007) 1225–1232.
[39] S. Asen, N.W. Stuart, H.W. Siegelman, Proc. Amer. Soc. Hort. Sci. 73 (1959) 495–501.
[40] Y. Cai, T.H. Lilley, E. Haslam, J. Chem. Soc., Chem. Commun. (1990) 380–383.
[41] T. Hoshino, U. Matsumoto, T. Goto, N. Harada, Tetrahedron Lett. 23 (1982) 433–436.
[42] M.O. Sinnokrot, C.D. Sherrill, J. Amer. Chem. Soc. 126 (2004) 7690–7697.
[43] P.F. da Silva, J.C. Lima, A.A. Freita, K. Shimizu, A.L. Macanita, F.H. Quina, J. Phys. Chem. A 109 (2005) 7329–7338.
[44] T. Goto, T. Kondo, Angewand. Chem.: Int. Ed. 30 (1991) 17–33.
[45] K. Yoshida, T. Kondo, T. Goto, Tetrahedron 48 (1992) 4313–4326.
[46] M. Shiona, N. Matsugaki, K. Takeda, Proc. Jpn. Acad., Ser. B 84 (2008) 452–456.
[47] K.E. Pharr, K.M. Mayhew, H.D. Schreiber, J. Undergrad. Chem. Res. 5 (2006) 49–53.
[48] S. Kunsage-Mate, E. Ortmann, L. Kollar, K. Szabo, M.P. Nikfardjam, J. Mol. Struct. 891 (2008) 471–474.