Achieving Complexity at the Bottom. 2,6-Bis(arylidene) cyclohexanones and Anthocyanins: The Same General Multistate of Species

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ABSTRACT: As in supramolecular chemistry, complexity could also be achieved through a bottom-up approach. Anthocyanins and related compounds such as the compound (E)-6-(dimethylamino)-4-(4-(dimethylamino)-2-hydroxybenzylidene)-1,2,3,4-tetrahydroxanthylum chloride (1), here reported, exhibit this type of complexity. The thermodynamics and kinetics of the complex multistate of species of compound 1 were studied by conventional and stopped-flow UV-visible spectrophotometry as well as by NMR. The system follows the same multistate of species of anthocyanins, except for the presence at moderately basic pH values of a species possessing a spiro carbon. The introduction of two dimethylamino substituents in positions 4 and 7, modulates deeply the thermodynamic and kinetics of the system. A beautiful pH-dependent palette of colors is obtained, including a blue flavilylium cation at unusually high pH values. The protonation of the dimethylamino substituents is the key aspect for explaining the details of the spiro opening kinetics. The system was fully characterized by representing the mole fraction distribution and the relative energy level diagram of all multistate species as a function of pH.

INTRODUCTION

Complexity is a necessary requirement in biological systems. Complexity can be achieved through a bottom-up approach, as in supramolecular chemistry, or by a multistate of chemical species reversibly interconverted by external stimuli, such as pH and light. We coined this last concept as molecular metamorphosis.1 While in supramolecular chemistry the complexity results from the higher dimension and diversity of the building blocks, in metamorphosis it is the number of species that gives the complexity. Anthocyanins and related compounds are a paradigm of metamorphosis. Anthocyanins are the molecules that give most of the red, purple, and blue colors of flowers and fruits. In particular, the blue color is the most intriguing one because anthocyanins in vitro only exhibit blue color in transient situations and at equilibrium have a very small percentage of the blue quinoidal base and the colorless hemiketal is the dominant species. Moreover, the intense blue ionized quinoidal base is not stable in common anthocyanins.2

The strategy used by nature to produce blue color from anthocyanins is the stabilization of the quinoidal bases (neutral and monoionized), Scheme 1.7 In the flower of Commelina communis, the blue color is achieved by stabilizing the ionized quinoidal base through a metalanthocyanin that consists in a supramolecular structure of anthocyanin/flavone/metal in a ratio 6:6:2, Scheme 2a. Another alternative used by nature to get blue color is the acylation of the anthocyanins’ sugars. In the case of the heavenly blue anthocyanin there are three acylated sugar units leading to a π—π stacking around positions 2 and 4 of the flavilylium core preventing in this way the hydration reaction, Scheme 2b.8

The synthetic strategies to prepare blue flavilylium derivatives have followed two vectors: (i) the use of amino substituents, (ii) introduction of a double bond between ring C and ring B, to get the so called styrylflavilylium compounds.7

In recent years, we have investigated some 2,6-bis-(arylidene)cyclohexanones, from which it is possible to obtain the respective styrylflavilylium cation at low pH values.8,9 These molecules are considered as curcumin analogs10 and their biologic activity has been very intensively studied.11

Supporting Information

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Scheme 1. Multistate of Species of Pelargonidin-3-glucoside in Acidic Medium

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\begin{align*}
\text{Quinoidal base (A)} & \rightarrow \text{Flavylum cation (AH\textsuperscript{+})} & \text{Hemiketal (B)} & \rightarrow \text{cis-chalcone (Cc)} \\
\text{Hydration} & \quad \text{Tautomerization} & \quad \text{Isomerization} \\
\kappa_1 & \quad \kappa_2 & \quad \kappa_3 \\
\text{Ionized species are formed by deprotonation of the hydroxyl substituents at higher pH values.}
\end{align*}
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Scheme 2. Two Main Strategies of Nature To Get the Blue Color: (a) Metalloanthocyanins; (b) π−π Stacking with Acylated Sugars

Scheme 3. General Kinetic Scheme for Compound 1

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\text{Through this work we did not find any experimental evidence for the cis analogs obtained from isomerization of the pendent arm. More species are obtained at higher pH values, by deprotonation of the hydroxyl substituents.}
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work, the compound (E)-6-(dimethylamino)-4-(4-(dimethylamino)-2-hydroxybenzilidene)-1,2,3,4-tetrahydroxanthylum chloride, henceforth named 1, Scheme 3 (AH\textsuperscript{+}), bearing two dimethylamino substituents in positions 7 and 4′ was prepared. The multistate of species of 2,6-bis(arylidene)cyclohexanones is very similar to the one of anthocyanins with the particularity that a new and interesting species that we identify by the name spiro, Scheme 3, is formed. In compound 1, it is possible to have cis−trans isomers in the pendent arm although in this compound we did not find any evidence for the cis isomers.

One characteristic of the anthocyanins and related compounds is the dramatic influence of the position and nature of the substituents on the pH-dependent mole fraction distribution of the multistate species. This fact has crucial implications on the pH-dependent color of these compounds and also on the identification of all the species that are responsible for the biological activity. Moreover, other chemical species can additionally be present in some specific cases as, for example, the flavanones in 2′-hydroxyflavylum compounds and, as mentioned above, the spiro in 2,6-bis(2-hydroxybenziliden)cyclohexanones.

In the next paragraph, the state of the art regarding the complex multistate of species of anthocyanins and related compounds will be summarized, to provide insight on the comprehension of the behavior of compound 1. The studies carried out during the eighties of the last century by McClelland in synthetic flavylum and Brouillard in anthocyanins, paved the way to the actual knowledge of...
the thermodynamic and kinetic properties of these compounds. Flavylium cation is the stable species at low pH values, because the back reactions that give flavylium cation from hemiketal (B) and from quinoidal base (A) are of second order and proportional to the proton concentration, Scheme 1. Raising the pH, the neutral species are formed (as in Scheme 1) but in basic medium ionized analogs are obtained from the deprotonation of the hydroxyl substituents.

The most convenient way to study the multistate of species of these systems is to perform direct and reverse pH jumps defined, respectively by the addition of base to solutions equilibrated at lower pH values (usually from flavylium cation at pH ≤ 1) and addition of acid to solutions equilibrated at higher pH values.

Some years ago, we introduced the energy level diagram for anthocyanins and related compounds in acidic medium,15 Scheme 4, and more recently extended it to the basic medium,16 see below. In such type of diagrams the energy level of several components is positioned, provided that the equilibrium constants (K) are calculated

\[
\Delta G^0 = -RT \ln K
\]

After a direct pH jump to sufficiently high pH values the quinoidal base is formed during the mixing time of the stopped flow. These two species (flavylium cation and quinoidal base) remain in equilibrium during the subsequent kinetic processes that are much slower. As shown in Scheme 4 the next step (2nd) is the hydration reaction (min) followed by the ring-opening of the hemiketal (ms) which is faster. By consequence, the second kinetic step is controlled by the hydration reaction. Finally, the system equilibrates through the isomerization that gives rise to a relatively small mole fraction of Ct, a process which usually takes a few hours, or even days.

In spite of the complexity of the anthocyanins multistate of species it can be reduced to a polyprotic system as shown in eqs 1–6.

\[
\begin{align*}
AH^+ + H_2O &\rightleftharpoons CB + H_3O^+ & K'_1 \\
CB + H_2O &\rightleftharpoons CB^- + H_3O^+ & K'_a \\
CB^- + H_2O &\rightleftharpoons CBH_2O^- + H_3O^+ & K''_a
\end{align*}
\]

where

\[
\begin{align*}
[CB] &= [A] + [B] + [C_c] + [C_t] \\
[CB^-] &= [A^-] + [B^-] + [C_c^-] + [C_t^-] \\
[CBH_2O^-] &= [A^{\text{H}^-}] + [B^{\text{H}^-}] + [C_c^{\text{H}^-}] + [C_t^{\text{H}^-}]
\end{align*}
\]

Pseudo-Equilibrium. When the cis–trans isomerization is slow, it is possible to define a transient state before formation of significant amounts of trans-chalcones, where all multistate species are in (pseudo-equilibrium). The following equations account for this state

\[
\begin{align*}
AH^+ + H_2O &\rightleftharpoons CB^\wedge + H_3O^+ & K'_\wedge \\
CB^\wedge + H_2O &\rightleftharpoons CB^{\wedge^-} + H_3O^+ & K'^{\wedge^-} \\
CB^{\wedge^-} + H_2O &\rightleftharpoons CBH_2O^{-\wedge} + H_3O^+ & K^{\wedge^{-\wedge}}
\end{align*}
\]

where

\[
\begin{align*}
[CB^\wedge] &= [A] + [B] + [C_c] \\
[CB^{\wedge^-}] &= [A^-] + [B^-] + [C_c^-] \\
[CBH_2O^{-\wedge}] &= [A^{\text{H}^-}] + [B^{\text{H}^-}] + [C_c^{\text{H}^-}]
\end{align*}
\]

Figure 1. Characterization of the pseudo-equilibrium of compound 1 (3 × 10⁻⁵ M) in H₂O/MeOH 1:1 (v/v); (a) pH dependent absorption spectra circa 1 min after a direct pH jump followed by a common spectrophotometer, ~0.2 < pH < 5.4; (b) the same for 6.0 < pH < 12.3; (c) fitting was achieved for a diprotic system with pK_{a1} = 1.9 and pK_{a2} = 7.5.
RESULTS AND DISCUSSION

Question of the Amine Protonation. The pH dependent absorption spectra of compound 1 taken circa 1 min after direct pH jumps are shown in Figure 1. The system is equivalent to a single diprotic acid with pK$_{a1}$ = 1.9 and pK$_{a2}$ = 7.5. In synthetic flavlyum compounds bearing amino substituents it was verified that the amino group can protonate at extremely high concentrations of proton in the range 1 M < pH < 6 M. The present compound, the absorption spectrum changes up to [H$^+$] = 0.6 M and remains the same for higher proton concentrations, Figure 1a. This result can be explained with two alternative hypotheses: (i) the dimethylamino group of flavlyum cation does not protonate under extremely acidic solutions; (ii) the absorption spectrum at 0.6 M corresponds to the protonated flavlyum and the "normal" flavlyum cation is the species observed at pH = 5.4 in Figure 1a. The fact that 1 has two donor amino substituents supports the second argument. Regardless of which amine is protonated (in principle the one in position 4' should protonate first because it is more distant from the pyrylium positive charge, Scheme 3), the presence of the other amino group (in position 7) renders its protonation easier (taking place at less acidic pHs) because of the delocalization of the positive charge. The first inflection point at pH = 1.9 in Figure 1a reflects the protonation equilibrium of the amine in the flavlyum cation. Therefore, the other inflection point at pH = 7.5 is equivalent to the pseudo-equilibrium defined in anthocyanins by eq 7, the state where AH$^+$ equilibrates with all the other neutral species, except the trans-chalcone. Equations 13–17 account for the pseudo-equilibrium in 1. In eq 16 the spiro form, SP, was also included. The spiro in 1 was obtained in a mixture of DMSO-d$_6$ (80%)/D$_2$O (20%) at pH 10 and characterized by NMR, see below. The absorption spectrum of the NMR solution (see Table S4 in Supporting Information) is identical to the absorption band centered at 300 nm of Figure 2b at higher pH values. We were not able to acquire the NMR spectra in the mixture MeOD/D$_2$O 1:1 (v/v) used throughout this work due to the low solubility of the compound at the concentrations needed to run the NMR experiments.

While the spectra of Figure 1a reflect the amine protonation of the flavlyum cation, eq 13, the spectra of Figure 1b are similar to those found in some anthocyanins and related compounds, although in this case this process occurs at higher pH values. It corresponds to the equilibrium between flavlyum cation and the species CB$^\gamma$, that includes at least the quinoidal base, A, and the spiro, SP, eqs 14 and 16.

\[
\begin{align*}
AH_2^{\gamma+} + H_2O &\rightleftharpoons AH^+ + H_3O^+ & K_a \\
AH^+ + H_2O &\rightleftharpoons CB^\gamma + H_3O^+ & K_a^{\gamma} \\
CB^\gamma + H_2O &\rightleftharpoons CB^\gamma^- + H_3O^+ & K_a^{\gamma^-}
\end{align*}
\]

where

\[
\begin{align*}
[CB^\gamma] = [A] + [B] + [Cc] + [SP] \\
[CB^\gamma^-] = [B^-] + [Cc^-]
\end{align*}
\]

In conclusion, according to Figure 1 the pseudo-equilibrium is defined by pK$_{a}$ = 1.9 (protonation of the amino group of the flavlyum cation) and pK$_{a2}$ = 7.5, the equilibrium between the flavlyum cation and the species that constitute CB$^\gamma$, eq 14. There is experimental evidence for the existence of A and spiro in CB$^\gamma$. Conversely, no spectral evidence for the formation of ionized species at the pseudo-equilibrium, eqs 15 and 17 was achieved.

Additional information from the absorption spectra monitored 10 ms after the addition of the base up to a few seconds was achieved by carrying out a series of direct pH jumps from pH = 1.0 to representative pH values, followed by the stopped-flow, Figure 2.

According to Figure 2a,b, in the pH range 1 < pH < 5.7 the protonated flavlyum cation and the flavlyum cation are formed initially in a ratio equal to [AH$^+$]/[AH$_2^{2+}$] = K$^{-\gamma}$/[H$^+$]. The proton transfer is the fastest reaction of the multistate and takes place during the mixing time of the stopped-flow. For pH jumps both to 1.9 and to 5.7, the absorption spectra obtained after 10 ms, after 10 s and after 1 min (not shown) are coincident. This is in accordance to the presence of AH$_2^{2+}$ and AH$^+$ prior to the reverse pH jump. Differently, the pH jump to pH = 9.7, Figure 2c, shows that the pseudo-equilibrium is

Figure 2. Spectral variations followed by stopped-flow after a direct pH jump of a solution of compound 1 (2.25 × 10$^{-5}$ M) in H$_2$O/MeOH 1:1 (v/v) at pH = 1 to the following pH values: (a) pH = 1.9; (b) pH = 5.7; and (c) pH = 9.7.
reached after 0.3 s. The species that is formed, with $\lambda_{\text{max}} = 304$ nm has the same absorption maximum as the spiro compound identified by NMR (Supporting Information, Table S4). This means that most presumably the spiro is obtained from intramolecular cyclization of the quinoidal base (i.e. formed immediately upon the pH jump to 9.7). However, we cannot exclude the possible pathway through the hemiketal. This result is similar to the behavior of anthocyanins, that is, the quinoidal base does not hydrate in acidic medium, but reacts with the hydroxide anion in basic medium to give the hemiketal, that later tautomerizes (ring opening) leading to Cc.

In Figure 3a, a direct pH jump to pH = 12.7 shows the initial formation of the quinoidal base that disappears to give an absorption band, the shape and position of which resemble those of a trans-chalcone (below we prove that it is Ct$^-$) together with an absorption in the UV characteristic of the spiro form ($\lambda_{\text{max}} = 304$ nm).

At the equilibrium, Figure 3b, the monoionized trans-chalcone is formed at higher pH values and a third $pK_a = 11.8$ is defined between the neutral species (A and spiro) and the monoionized trans-chalcone (Ct$^-$), see Supporting Information, Table S5. Only at high pH values (pH > 10), the pseudo-equilibrium and the equilibrium become different. In Scheme 5 the mole fraction distribution of species at the pseudo-equilibrium and equilibrium are shown. The color palette obtained from equilibrated solution of compound 1 is also presented.

NMR Experiments. The study of compound 1 was complemented by running NMR spectra at different pH values (Figure 4). A sample for NMR was prepared by dissolving the synthesized compound 1 in 450 $\mu$L of DMSO-$d_6$ and 50 $\mu$L of deuterated trifluoroacetic acid (TFA). The $^1$H NMR spectra were run at 298 K and any evolution of the system followed over time. The $^1$H NMR spectrum only shows one set of peaks corresponding to a single flavylum: two singlets assigned to protons 4 and 1’b (Table S1, Supporting Information); two ABX sets corresponding to three protons in relative positions 1, 2, and 4 in the same aromatic ring, a set of aliphatic protons corresponding to the propylene bridge and two aliphatic singlets corresponding to amine methyl groups. The singlet at the lowest field was assigned to H4 on the basis of the expected lowest electron density on this position and in accordance with many published flavylum derivatives. Full characterization and assignment of $^1$H and $^{13}$C signals was achieved with heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) and COSY spectra (Table S1, Supporting Information) allowing identification of the corresponding monoprotonated flavylum trans isomer, most probably at the 4” position (AH2$^+2s$). The same NMR experiment was performed modifying the solvent to the same one used in UV−vis spectroscopic measurements (CD3OD/D2O/DCl 1:1:0.05). The $^1$H NMR spectra were run at 298 K and showed again the same set of peaks previously observed in DMSO-$d_6$/TFA (9:1)
According to the nuclear Overhauser enhancement spectroscopy (NOESY) correlation between the methylene aliphatic protons and H6\(^{\text{a+}}\), the presence of the mono protonated flavylum \textit{trans} isomer is confirmed (Figure 4, pink spectrum, \(\text{AH}_2^{2+}\), and Table S2, Supporting Information).

The extremely low solubility of the compound 1 in MeOH/H\(_2\)O 1:1 limited the use of NMR and mixtures of DMSO/D\(_2\)O had to be used. Compound 1 was dissolved in a mixture of DMSO-d\(_6\)/D\(_2\)O (8:2) and the pD was adjusted to 6.0 with deuterated acetate buffer (0.025 M). The UV–vis spectra in this mixture of solvent is coincident with the corresponding spectra at pH 5.2 in MeOH/H\(_2\)O 1:1 (Table S3, Supporting Information).

Full characterization and assignment of \(^1\)H and \(^13\)C signals was achieved with HSQC, HMBC, COSY, and NOESY spectra (Table S3, Supporting Information), allowing us to identify the corresponding \textit{trans} flavylum isomer (\(\text{AH}^+\)) (blue spectrum, Figure 4).

To characterize the species present under basic conditions, compound 1 was dissolved in a mixture of DMSO-d\(_6\)/D\(_2\)O (8:2) and the pD adjusted to 10.0 with a 0.1 M NaOD solution in D\(_2\)O. The UV–vis spectrum in this solvent mixture is coincident with the corresponding UV–vis spectrum at pH 10.0 in MeOH/H\(_2\)O 1:1, except for the absence of the structured band in the visible (Table S4, Supporting Information). The low solubility of the compound in DMSO-d\(_6\)/D\(_2\)O (8:2) limited the use of NMR. It was nevertheless possible to acquire a \(^1\)H NMR spectrum that showed only 4 aromatic signals, a clear indication that the structure is very symmetric (Table S4, Supporting Information) and could allow us to suggest a spiropyran-like structure which most likely corresponds to the spiro (Sp) species (Figure 4, black spectrum).
Compound 1 was further dissolved in a mixture of DMSO-d_6/NaOD (2 M), and the respective ¹H NMR showed only 4 aromatic signals, (red spectrum, Figure 4, Table S5, Supporting Information), an indication of an ionized trans-chalcone. The respective UV−vis spectrum is 30 nm red shifted with the corresponding UV−vis spectrum at pH 12.7 in MeOH/H_2O 1:1 (Table S5, Supporting Information). Most probably in both solvents in basic medium we are dealing with the monoionized species; a red shift of 20 nm was also observed for the flavlylium cation at pH = 1.0 from DMSO/H_2O 1:1 to MeOH/H_2O 1:1.

Reverse pH Jumps. Reverse pH jumps from solutions equilibrated at different pH values during 1 day to pH = 1.0 are presented in Figure 5a. In the pH range from 1 < pH < 5 the flavlylium is the sole observed species with a more or less constant absorption spectrum. This result suggests that after 1 day the solutions in this pH range (at the pseudo-equilibrium) are flavlylium cations (protonated and unprotonated at the amino substituent). In the pH range 7 < pH < 10.5 the flavlylium cation is still the sole observed species but its absorption grows with a monoexponential kinetics. In this initial pH range the flavlylium cation is equilibrated with quinoidal base and spiro forms. The quinoidal base existing prior to the pH jump (in equilibrium with some AH⁺ for lower pHs) corresponds to the initial flavlylium cation, and the monoexponential growth is due to the formation of more flavlylium from the spiro. However, we cannot exclude the existence of some minor fractions of cis-chalcone and hemiketal, in spite of the monoexponential nature of the trace.

At pH = 11.7, the formation of an absorption spectrum that could be attributed to a monoprotonated trans-chalcone on the basis of the observed λ_max = 348 nm is clear. The spectral evidence for the trans-chalcones is given in Figure 5b. The solution at pH = 12.6 was reverted back to a series of different pH values. Only three different absorption spectra could be identified, corresponding to Ct⁺, Ct and Ct⁻.

The diagram of Scheme 6 permits to rationalize not only the thermodynamic behavior but also the kinetic steps. A direct pH jump to pH = 5.0 gives exclusively the (non-protonated) flavlylium cation. In the case of a pH jump to 10.0, the quinoidal base is immediately formed but in a few sub-seconds equilibrates with the spiro. When a direct pH jump is performed to pH = 12.0 the most stable species is now Ct⁻; the kinetics is slow because the spiro should open in a cis form that has to isomerize. The isomerization can be slow by intrinsic reasons or if the energy level of the cis species is much higher than the respective trans. We were not able to position the cis forms in the energy level diagram because of their transient nature.

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Spiro Opening. A series of reverse pH jumps from equilibrated solutions at pH = 10.1 back to lower pH values was performed and the rate constants toward the new equilibrium represented in Figure 6.

At pH = 10 there is an equilibrium between the spiro and the quinoidal base. The reverse pH jumps to pH = 1.0, Figure 6a, show the formation of the two flavlylium cations with predominance of the amino protonated one. The spectrum
after 10 ms results from the conversion of the quinoidal base into the flavlylium cations. The kinetic process is mono-exponential with a rate constant \( k_{\text{obs}} = 2.5 \text{ s}^{-1} \), reflecting the kinetics of the spiro opening. The intermediate from the spiro opening should be a cis form, while the observed flavlylium cations are in the trans geometry. This confirms that the cis–trans isomerization in this compound seems to be very fast and consequently we only observed experimentally the trans forms.

The absorption spectra taken 10 ms after the reverse pH jump, to several lower pH values, Figure 6b, shows the quinoidal base or the flavlylium cations according to the final pH value. Besides the quinoidal base and the flavlylium cation absorptions in the visible, there are absorption bands in the UV region of the spectrum characteristic of the spiro form. Interestingly, the transient forms of the protonated spiro appear at lower pH values and the absorption versus pH is compatible with two species with inflection points at pH = 2.6 and pH = 4.2. We assign these pH values to the first and second protonations of the spiro (at the amino groups). From this point onwards, the system evolves to the equilibrium with a rate constant that changes with pH as represented in Figure 6c. The shape of Figure 6c can be fitted with eq 18.

\[
k_{\text{obs}} = k_1 X_{\text{SP}^2+} + k_2 X_{\text{SP}^+} + k_3 X_{\text{SP}} + k_{\text{OH}} [\text{OH}^-]
\]

where \( X \) represents the mole fractions of the three spiro species, \( k_1, k_2 \) the respective rate constants and \( k_{\text{OH}} \) the rate for the basic catalysis, similarly to the one observed in hemiketals derived from flavlylium cations.\(^{16} \) The fitting was achieved for the following parameters: \( k_1 = 1 \text{ s}^{-1} \); \( k_2 = 75 \text{ s}^{-1} \); \( k_3 = 15 \text{ s}^{-1} \); \( k_{\text{OH}} = 1.0 \times 10^7 M^{-1} \text{ s}^{-1} \).

According to the kinetic data of the spiro opening reported in Figure 6c, four different regimes could be identified as a function of pH: (i) highly acidic (pH < 2.6) where the SP\(^{2+}\) species is the dominant one, (ii) acidic (2.6 < pH < 4.2) where the SP\(^+\) species prevails, (iii) neutral (4.2 < pH < 7.2) where the neutral SP is the representative species and (iv) the basic one (pH > 7.2) where the rate increases with increasing pH.

The fitting through eq 18 indicates (i) the fully protonated SP\(^{2+}\) species exhibits the slower rate, (ii) the highest rate takes place for the monoprotonated form, and (iii) the rate decreases again in the case of the nonprotonated spiro. We suggest, Scheme 7, that the spiro opening is controlled by the possibility of the nitrogen atoms to share their lone pair to the conjugated \( \pi \)-system and also by the electrophilicity of the...
ketonic carbon of spiro (C2). In that sense, it seems reasonable to suggest that the more reactive spiro species will be the one able to maximize both requirements. The monoprotonated spiro form (2.6 < pH < 4.2) has a nitrogen atom with a donating lone pair available, and the ketonic carbon shows an important electrophilic character because of the protonation of the other amino group. For that reason we consider plausible that this species presents the higher rate constant observed (k1 = 75 s⁻¹). In the neutral spiro form (pH > 4.2) both nitrogen atoms present their lone pair available, however the electrophilic character of the ketonic carbon is lower and therefore the kinetic rate decreases (k1 = 15 s⁻¹). Finally, the diprotonated spiro species (pH < 2.6) presents the higher electrophilic character in its ketonic carbon; however, none of the lone pair of nitrogen atoms are available to promote the intramolecular spiro opening. In this case, we propose that the spiro opening is due to a nucleophilic attack of a water molecule to its ketonic carbon. Therefore, the process is intermolecular instead of intramolecular and it is controlled by the diffusion of a water molecule. This change in the molecularity of the reaction induces a drastic reduction on the constant rates observed (k1 = 1 s⁻¹).

■ CONCLUSIONS

The results achieved through this work confirm that the multistate of anthocyanins is not restricted to these compounds and could be the general behavior of all flavilium derivatives. The scientific approach and the knowledge developed for anthocyanins multistate of species during more than one century of research is a powerful tool that permitted rationalizing the complex 2,6-bis(arylidenecyclohexanones multistate of species.

In this particular case, the presence of two amino substituents in position 7 and 4’, lead to a push–pull effect from both amino groups to the pyrylium core, allowing the stabilization of the blue flavilium cation up to pH = 8, a feature not shown in conventional synthetic flavilium compounds, where Ct becomes the most stable species (typically from pH values over 4 or 5). Another important aspect in 2,6-bis(arylidenecyclohexanones is the presence of the spiro form that is the equivalent of the hemiketal in anthocyanins, which seems to be stabilized by the propylenic bridge.

■ EXPERIMENTAL SECTION

Materials and Methods. All chemicals and solvents employed for synthesis and preparation of samples were of analytical grade and used without further purification if not otherwise specified. The NMR spectra were recorded on a Bruker ADVANCE III 400 spectrometer (400 MHz for 1H, 100 MHz for 13C) at 298 K. NMR assignments have been carried out on the basis of 1D NMR spectra (1H, 13C, DEPT 135) and 2D NMR spectra (NOESY, COSY, HSCQ and HMBC). Elemental analysis was performed on an elemental analysis system vario MICRO cube from Elementar Analyssensysteme GmbH.

Synthesis of 6-(Dimethylamino)-4-(4-(dimethylamino)-2-hydroxybenylidene)-1,2,3,4-tetrahydroxanthylum (1). The compounds 4-dimethylamino-2-hydroxybenzaldehyde (0.39 g, 2.36 mmol) and cyclohexanone (0.116 g, 1.18 mmol) were dissolved in 10 mL of ethanol and heated to 50 °C. The mixture was stirred while dry hydrogen chloride gas was generated and bubbled in the mixture. After 3 h of stirring, the color of the solution was changed from yellow to purple. The reaction finished after 24 h. The precipitate that formed was filtered off, recrystallized from methanol/diethyl ether, and washed with diethylether. A dark violet powder was obtained (60% yield).

Elemental analysis for C24H29Cl3N2O2·HClO4 (60% yield). Calculated: C, 58.2; H, 6.45; N, 5.43%. Found: C, 58.5; H, 6.53; N, 5.62%.

Thermodynamic and Kinetic Studies. The pH jumps were carried out by adding a stock solution of flavilium salt in 1:1 MeOH/HCl 0.2 M (1 mL) to a 3 mL quartz cuvette containing a solution of 1:1 MeOH/NaOH 0.2 M (1 mL), MeOH (0.5 mL), and universal buffer of Theorell and Stenhagen (0.5 mL) at the desired final pH. This defined the ionic strength as 0.1 M (controlled by the NaCl concentration resulting from neutralization). The final pH of the solutions was measured in a Crison basic 20 + pH meter. Spectroscopic measurements were performed using Milli-Q water and methanol HPLC grade, with a constant temperature of 20 ± 1 °C, with a Varian-Cary 100 Bio or Varian-Cary 5000 spectrophotometers. The stopped-flow experiments were conducted in an Applied Photophysics SX20 stopped-flow spectrometer provided with a PDA.1/UV photodiode array detector. The NMR and absorption spectrum at basic medium was carried out in a saturated solution of compound 1 in (0.7 mL DMSO-d6 + 0.08 mL NaOD 40% + 0.1 mL D2O) circa 0.9 M in base.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02745.

Copies of 1H, 13C, and 2D NMR spectra and full peak assignment of compound 1 at different pDs (PDF)

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Notes

The authors declare no competing financial interest.

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“R = perfect gases constant; T = temperature in kelvin; \( \Delta G^0 \) = free Gibbs energy.

The spectrum cannot distinguish between C(2+) or C(1+), if the proton is in fast exchange between the two hydroxyl groups.

The stopped flow cannot be used for these extremely acidic solutions and the reaction to give the protonated flavilyum is very fast to get a good absorption spectrum.

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