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Physicochemical Properties of Fluorescent Probes: Experimental and Computational Determination of the Overlapping pK_a Values of Carboxyfluorescein

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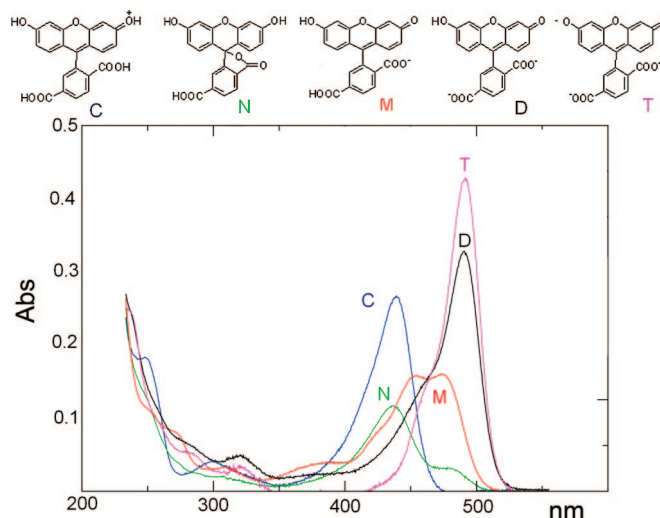
The overlapping pK_a values of carboxyfluorescein have been measured by applying a UV–vis spectroscopic method that uses absorbance diagrams and chemometric analysis. Quantum chemical calculations have also been performed in order to analyze the experimental data.

Introduction

Fluorescent probes (FP) are a class of molecules widely used for analyzing, at the molecular level, very complex systems ranging from membrane to biomacromolecules.

The basic feature of a FP is its polarity-dependent spectral response which can be connected to local structures and motions. In this respect, an exhaustive physicochemical characterization represents an important prerequisite for a given molecular system to be used as FP. As a matter of fact, alteration of spectral response may also derive from intrinsic structural changes which, therefore, should be predictable and controllable as much as possible.

6-Carboxyfluorescein (CF, see Figure 1) is a very common fluorescent probe. Its very high molar absorptivity at the wavelength of the argon laser (488 nm), high fluorescence quantum yield,[1] and the presence of reactive functional groups make CF a useful and sensitive fluorescent label. In particular, CF and its derivatives have been widely used in biological, pharmaceutical, and biochemical applications such as fluorescent protein labeling[2] and imaging,[3,4] as detectors of helminticide activities,[5] as cargo for membrane penetration,[6] as guest-

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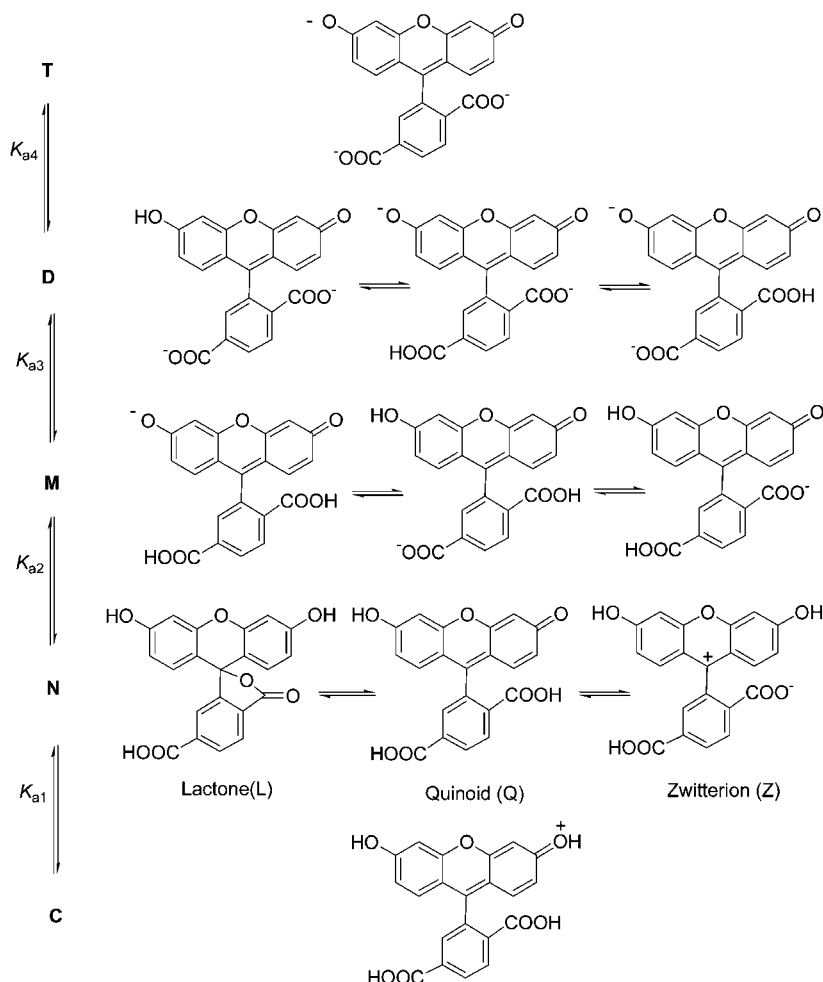
FIGURE 1. Prototropic equilibria of CF. Tautomerization and macroscopic acid dissociation equilibria are highlighted.

indicators in chemosensors,[7] and as acceptors in intramolecular fluorescence energy transfer (FRET) compounds.[8] CF is also the most common fluorescent detector of liposome lysis allowing the determination of liposome stability.[9–11] In this application, CF has completely superseded fluorescein due to the lower permeability of the former in the bilayer.[12] In a typical experiment, the dye is entrapped inside lipid vesicles in a high, self-quenched concentration (ca. 50 mM). Efflux of CF from the inner aqueous compartment of liposomes results in a dilution of the dye into the surrounding medium and reestablishment of fluorescence.[13]

In aqueous solution, CF can exist in one or more of four different acid/base pairs (cation, neutral, monoanion, dianion,

and trianion) depending on the pH.[14,15] However physicochemical and structural definition of such a molecule is still incomplete. For example, accurate evaluation of CF pK_a values, to the best of our knowledge, has never been addressed. The lack of such information, which has not prevented in the last years its massive use as an acid–base indicator,[16,17] might sometimes seriously affect the interpretation of thermodynamic and/or spectroscopic data which must rely on empirical considerations. Today, joint application of experimental and computational methods allows access to extremely accurate physicochemical properties even in relatively complex systems such as aqueous CF. Therefore, inspired by recent studies[18,19] on a CF analogue, e.g., fluorescein, we decided to evaluate the pK_a's of CF as a starting point for future studies aimed at its exhaustive characterization.

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TABLE 1.   pKa Values Determined by UV–Vis Determinations, Chemometric Analyses, and Quantum-Chemical Calculations

	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
UV–vis determinations[a]	2.4 ± 0.3	4.2 ± 0.2	4.9 ± 0.1	6.7 ± 0.1
chemometric analyses[b]	1.9 ± 0.1	4.1 ± 0.1	6.0 ± 0.4	7.0 ± 0.5
quantum-chemical calculations	1	5	6	13

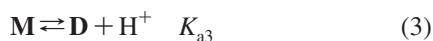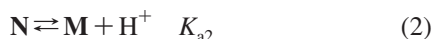[a] Errors are calculated as standard errors of the average values. [b] See ref 21.
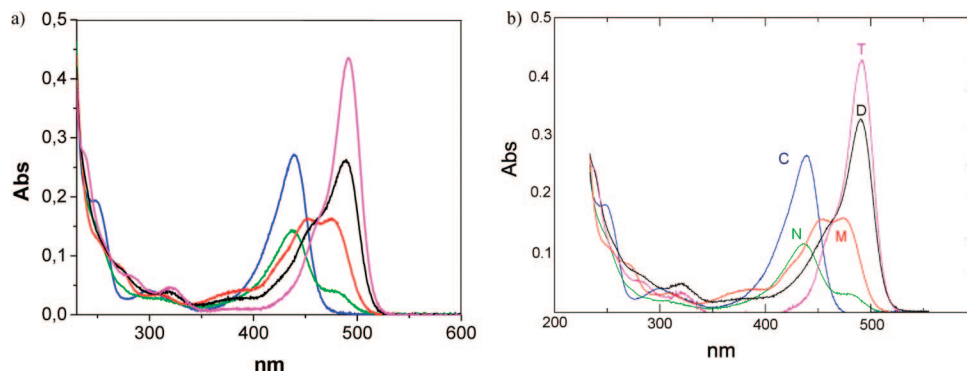
FIGURE 2. (a) UV–vis spectra recorded at pH 1.09 (blue), 2.96 (green), 5.04 (red), 6.39 (black), and 7.69 (magenta). (b) Spectra of the cation (blue), neutral (green), monoanion (red), dianion (black), and trianion (magenta) obtained from chemometric analysis.

Spectrophotometric titration is a simple method to measure pK_a values of acids, provided that the investigated molecule possesses a chromophore in the proximity of the ionization center. Nevertheless, acid dissociation constants relating the concentrations of protolytic forms whose spectra overlap substantially and/or possess overlapping pK_a values are difficult to determine using conventional spectrophotometric titration.

In this work, we have spectrophotometrically determined the four overlapping pK_a values of CF in water by using both absorbance diagrams[20] and chemometric analysis.[18] Moreover, in order to explain and confirm the pK_a values obtained, we have carried out quantum-chemical calculations in the mean-field approximation which have been widely adopted for this purpose in recent years.[19]

Results

Chemometric Analysis. This analysis was performed in order to obtain indicative pK_a values of CF, which could be used as input for the following determination using absorbance diagrams. Chemometric analysis of the spectroscopic data has been carried out with methods already described in the literature[18] and considering the following multicomponent model of CF acid–base equilibria in water:

$$\mathbf{C} \rightleftarrows \mathbf{N} + \mathrm{H}^+ \quad K_{a1} \qquad (1)$$

$$\mathbf{N} \rightleftarrows \mathbf{M} + \mathrm{H}^+ \quad K_{a2} \qquad (2)$$

$$\mathbf{M} \rightleftarrows \mathbf{D} + \mathrm{H}^+ \quad K_{a3} \qquad (3)$$

$$\mathbf{D} \rightleftarrows \mathbf{T} + \mathrm{H}^+ \quad K_{a4} \qquad (4)$$

where **C**, **N**, **M**, **D**, and **T** refer, respectively, to the cationic, neutral, monoanionic, dianionic, and trianionic forms of CF reported in Figure 1.

The aspects which are relevant for the case under study are reported in the Experimental Section. The chemometric calcula-
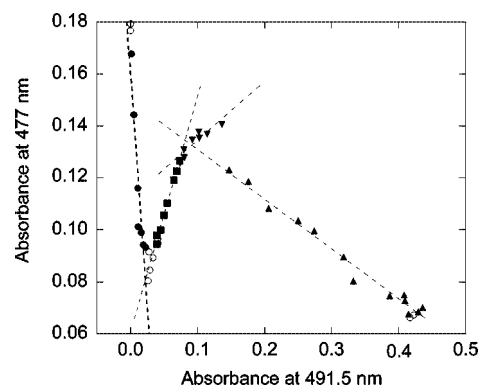
FIGURE 3. Absorbance diagram of CF, pH range 1.1–9.3. The lines refer to the pH intervals reported below and which correspond to the four equilibria of the CF. The reaction described in eq 1 predominates in the 1.5–3.2 pH range (●), the second equilibrium (eq 2) predominates in the 3.9–4.7 pH range (■), the third equilibrium (eq 3) predominates in the 4.9–5.7 pH interval (▼), and finally, the fourth equilibrium (eq 4) predominates between pH 6.0 and 9.3 (△).
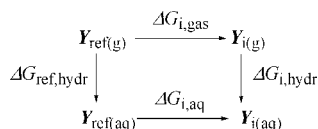
tions were performed using homemade routines previously tested on literature data[18,21]

The data obtained by chemometric analysis are reported in Table 1 and in Figure 2. Note that for the estimation of the error in the chemometric analyses we have followed the same method as reported in the literature.[21] Figure 2a reports the spectra of samples recorded at pH values which should ensure, according to the pH interval derived from the A diagram of Figure 3 (see the following section), the prevailing, even if not sole, presence in the solution of the marked species. Figure 2b reports the spectral responses evaluated by using the chemometric model.

It is interesting to observe that the chemometric signal derived for **D** appears overestimated by ca. 0.1 absorbance units with respect to the corresponding signal at pH 6.39. Such a discrepancy parallels the rather poor agreement between the pK_{a3} values determined by the two methods.

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SCHEME 1. Generic Thermodynamic Cycle for the Calculation of Reaction Free Energy in Water

$$Y_{ref(g)} \xrightarrow{\Delta G_{i,gas}} Y_{i(g)}$$

$$\Delta G_{ref,hydr} \downarrow \qquad \qquad \downarrow \Delta G_{i,hydr}$$

$$Y_{ref(aq)} \xrightarrow{\Delta G_{i,aq}} Y_{i(aq)}$$

SCHEME 2. Thermodynamic Cycle Used for the Calculation of Reaction Free Energy in Water between Selected Isomers (Reference) of the Acid/Base Pair

$$AH_{(g)} \xrightarrow{\Delta G_{AH/A^-,gas}} A^-_{(g)} + H^+_{(g)}$$

$$\Delta G_{AH,hydr} \downarrow \quad \Delta G_{A^-,hydr} \downarrow \quad \downarrow \Delta G_{H^-,hydr}$$

$$AH_{(aq)} \xrightarrow{\Delta G_{AH/A^-,aq}} A^-_{(aq)} + H^+_{(aq)}$$

Absorbance Diagrams. A second experimental spectrophotometric determination was performed by recording a higher number of spectra in the proximity of the pH values corresponding to the indicative pK_a values obtained by chemometric analysis.

In order to obtain the four pK_a values, we plotted the absorbance of the CF solution at a specified pH at one wavelength against the absorbance at a second wavelength at the same pH. The so obtained A diagram (or absorbance diagram)[20,22] shows the changes of the absorbance of CF at two wavelengths as a function of the pH of the buffer solutions. As the absorbance of a dissolved molecule at any wavelength is proportional to the concentration of that species, the A diagram should be linear if only one species or one equilibrium is present in the solution. Nevertheless, the slope of the line of the A diagram is expected to vary or change direction every time a new equilibrium becomes dominant in the system.

An absorbance diagram (A diagram) was drawn by using experimental spectrophotometric data at λ 447 and 491.5 nm (see Figure 3). The choice of the wavelengths was done in the light of the spectra of the individual species obtained by the chemometric analysis (see Figure 2b) in order to simplify the system under investigation.[20] At a selected wavelength of 491.5 nm, the absorption of the trianion and dianion is maximum while that of the cation is null. At this wavelength, **N** and **M** show an intermediate absorption (even if the absorbance of the neutral form is relatively low). On the other hand, at 447 nm (the second wavelength selected) all of the acid/base species absorb.

The four pK_a values of CF obtained by A diagrams are reported in Table 1.

In the pH intervals 1.5–3.2 and 5.0–8.0, where it was possible to isolate in the solution only one acid/base pair (cation/neutral form in the former, dianion/trianion in the latter pH interval), the alternative classical spectrophotometric titration of the cation and of the dianion was also performed by plotting the absorbance at the wavelength of 437 and 491.5 nm, respectively vs pH.

Quantum Mechanical Estimation of pK_a Values. Quantum chemical estimation of pK_a of CF was evaluated in a rather standard fashion as reported in the Supporting Information. Beyond the well-known difficulties for computationally describing the thermodynamic properties of solvation,[23] the computational determination of the pK_a values is complicated, in the present case, by the fact that each acid/base pair may actually exist as an ensemble of isomers in a multiple tautomeric equilibrium (see the Supporting Information).

Once the reference isomer, Y_{ref}, is selected, we can calculate $\Delta G_{i,aq}$, i.e., the (standard molar) free energy associated to the conversion of Y_{ref} to the generic Y_i isomer using a Born–Haber thermodynamic cycle (Scheme 1).
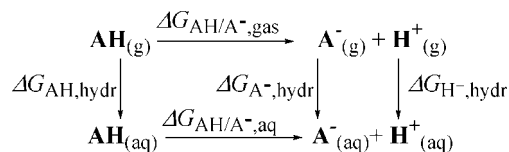
The terms in this cycle, at least in principle, may be calculated by quantum-chemical methods. However, the following approximations have been adopted according to optimized protocols:[19]

(i) The evaluation of hydration free energy ($\Delta G_{ref,hydr}$ and $\Delta G_{i,hydr}$) is carried out in the framework of mean-field approaches[24,25] such as the polarizable continuum model (PCM). This procedure represents a rather crude description of the solvent, but it is nowadays widely adopted for obtaining approximate evaluations of thermodynamic properties at infinite dilution. In the present case, conductor-like-PCM (C-PCM)[26] calculations were carried out within Hartree−Fock (HF) theory using the 6-31+G(d) basis set. Note that a geometry relaxation (maximum force lower than 0.01 au) was carried out in the presence of the mean-field for all of the structures.

(ii) The evaluation of the gas-phase reaction free energies was performed using density functional theory (DFT) with the PBE1PBE functional and the 6-311+G(2d,2p) basis set following a PBE1PBE/6-31+G(d) geometry optimization.

Molecular (vibrational) partition functions (Q_{vib}) in vacuo were calculated in the harmonic approximation in order to evaluate the term $-RT \ln Q_{vib}$ to be added to gas-phase free energies ($\Delta G_{i,gas}$).

Within the present computational scheme, reaction free energy in water is then evaluated using the formula

$$\Delta G_{i,aq} = G_{i,gas} + G_{i,hydr} - G_{ref,hydr} \qquad (5)$$

Similarly, the acid–base equilibrium between the reference isomers in the acid/base pair can be addressed by using the thermodynamic cycle of Scheme 2.

Therefore in the following equation (eq 6)

$$\Delta G_{AH/A-,aq} = \Delta G_{AH/A-,gas} + \Delta G_{A-,hydr} - \Delta G_{AH,hydr} + \Delta G_{H+,hydr} \qquad (6)$$

all of the terms were calculated as previously described, and for $\Delta G_{H+,hydr}$ we used the recommended value of −264.0 kcal/mol.[27]

The data obtained by quantum mechanical estimation are also reported in Table 1.

Discussion

In aqueous solutions, CF is involved in the four acid–base equilibria (1)–(4), depending on the pH. From the absorbance diagram of Figure 3 it is possible to locate, in the wide pH range investigated, four different linear segments corresponding to four different pH intervals in which only one acid–base

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TABLE 2. Experimental Ionization Constants of CF Determined by UV–vis Spectrophotometry

λ (nm)	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
		A Diagrams		
447.0	2.36 ± 0.05	4.06 ± 0.04	4.86 ± 0.06^a	6.61 ± 0.02
491.5	2.72 ± 0.05	4.36 ± 0.03^b	5.05 ± 0.05^c	6.70 ± 0.02^d
		UV–vis spectrophotometric titration		
	2.15 ± 0.04			6.63 ± 0.04

[a] Determined in the pH interval 4.9–5.5. [b] Determined in the pH interval 4.0–4.7. [c] Determined in the pH interval 4.9–5.4. [d] Determined in the pH interval 6.2–9.3.

TABLE 3. Calculated and Experimentally Obtained A_λ Values for the Cationic (C), Neutral (N), Monoanionic (M), Dianionic (D), and Trianionic (T) Forms of CF

λ (nm)	C^a	N	M	D	T
447.0	0.179	0.072	0.140	0.137	$0.067 (0.067)^b$
491.5	0.000	0.029	0.094	0.098	$0.434 (0.423)^b$

[a] Experimental values at pH 1.07. [b] Experimental value calculated as the average of the absorbance at pHs 9.34, 9.55, and 9.74.

equilibrium prevails. Spectrophotometric data are collected in Table S2 in the Supporting Information.

To obtain K_{a1}, the points located in the 1.5–3.2 pH range have been treated with the following equation 7:

$$(A_\lambda - A_{\lambda C})10^{-pH} = -K_{a1}A_\lambda + K_{a1}A_{\lambda N} \quad (7)$$

where A_λ is the absorbance due to the combined presence in the buffer solution of the cationic and neutral forms, at the selected wavelength λ, $A_{\lambda C}$ is the absorbance of the cationic form, and $A_{\lambda N}$ is the absorbance of the corresponding conjugate neutral base, **N**. The value of $A_{\lambda C}$ was experimentally determined at pH 1.4 where only the cationic form of CF is presumably present in the solution.

The line obtained by plotting $(A_\lambda - A_{\lambda C})10^{-pH}$ vs A_λ at λ 447 nm provides K_{a1} as the slope and $K_{a1}A_{\lambda N}$ as the intercept. By linear regression, the following values were obtained: $pK_{a1} = 2.36$; $A_{477 N} = 0.072$.

When the absorbances measured at λ 491.5 nm were plotted according to eq 7, the values obtained were as follows: $pK_{a1} = 2.72$; $A_{491.5 N} = 0.029$.

Similarly, $pK_{a2} = 4.06$ and $pK_{a2} = 4.36$, $pK_{a3} = 4.85$ and $pK_{a3} = 5.02$, $pK_{a4} = 6.61$ and $pK_{a4} = 6.70$ were obtained by using data recorded at λ 447 nm and at λ 491.5 nm, respectively. The values of $A_{447 M} = 0.140$ and $A_{491.5 M} = 0.094$, and $A_{447 D} = 0.137$ and $A_{491.5 D} = 0.098$, which are not experimentally detectable due to the impossibility to isolate in solution the acid/base pairs **N/M** or **M/D**, were analogously obtained. The equations used were as follows:

$$(A_\lambda - A_{\lambda N})10^{-pH} = -K_{a2}A_\lambda + K_{a2}A_{\lambda M} \quad (8)$$

$$(A_\lambda - A_{\lambda M})10^{-pH} = K_{a3}A_\lambda - K_{a3}A_{\lambda D} \quad (9)$$

$$(A_\lambda - A_{\lambda D})10^{-pH} = -K_{a4}A_\lambda + K_{a4}A_{\lambda T} \quad (10)$$

The data obtained are summarized in Tables 2 and 3.

All of the correlations referring to eqs 7–10 were constructed using at least six data points except in the case of eq 9, for data collected at 491.5 nm, where only five data points were used. The correlation coefficients obtained were >0.985, the only exceptions being the correlations of pK_{a1} ($r = 0.974$ with data collected at λ 447 nm and $r = 0.975$ with data collected at λ 491.5 nm) and of pK_{a3} ($r = 0.962$ with data collected at λ 447 nm and $r = 0.971$ with data collected at λ 491.5 nm). The
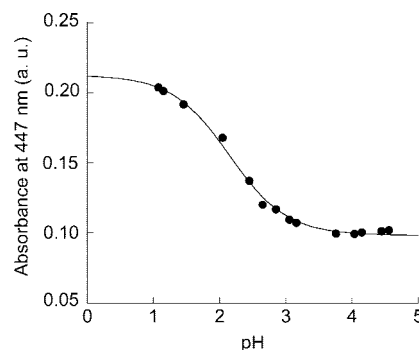
FIGURE 4. Plot of the absorption at 437 nm against the pH, in the pH interval 1.07 – 3.16, fitted into eq 11 (solid line).

agreement between the obtained pK_a values measured at different λ (see Table 2) and that of the experimental and the calculated $A_{\lambda T}$ values (see Table 3) demonstrates the reliability of the determination.

The values of K_{a1} can also be measured directly by UV–vis spectrophotometric titration as in the pH interval 1.07–3.16 the absorbance of the solution is essentially due to the cation/neutral acid/base pair and these two forms have a different absorbance at 437 nm. Assuming that both the acid and the base contribute to the observed absorbance (A_{tot}), eq 11 can be derived:

$$A_{tot} = A_C + A_N = \epsilon_C \frac{[CF]_{tot}K_{a1}}{K_{a1} + [H^+]} + \epsilon_N \frac{[CF]_{tot}[H^+]}{K_{a1} + [H^+]} \quad (11)$$

where $[CF]_{tot}$ is the total concentration of CF and ϵ_C and ϵ_N are the molar absorptivities of the cation **C** and the conjugate base **N**, respectively. From a best fit of experimental A_{tot} values to eq 10 the values of pK_{a1} 2.15 ± 0.04, ϵ_C 42521 ± 523 dm³ mol⁻¹ cm⁻¹, and ϵ_N 19689 ± 290 dm³ mol⁻¹ cm⁻¹ ($r = 0.996$) were obtained (see Figure 4).

Similarly, pK_{a4} 6.63 ± 0.04, ϵ_D 17733 ± 1791 dm³ mol⁻¹ cm⁻¹, and ϵ_T 84930 ± 739 dm³ mol⁻¹ cm⁻¹ ($r = 0.998$) were obtained from absorbance data at 491.5 nm in the pH interval 5.73 – 9.74 (see Figure S1 of the Supporting Information).

The values of pK_{a1}, pK_{a2}, and pK_{a4} are in good agreement with those reported[18,28] for fluorescein, which lacks the second carboxy group in position 6. In particular, the experimentally determined $pK_{a1} = 2.4 \pm 0.3$ differs by $+0.3^{18}$ and $+0.2^{28}$ pK units from published data determined at the same ionic strength (0.1 M). The experimentally determined $pK_{a2} = 4.2 \pm 0.2$ differs by only -0.1^{18} and -0.2 units,[28] while the obtained $pK_{a4} = 6.7 \pm 0.1$ differs by $+0.3$ units[18,28] from published data.

The obtained value of pK_{a3} is 4.9 ± 0.1, and it is the value expected for a carboxy group in para position to the first one in

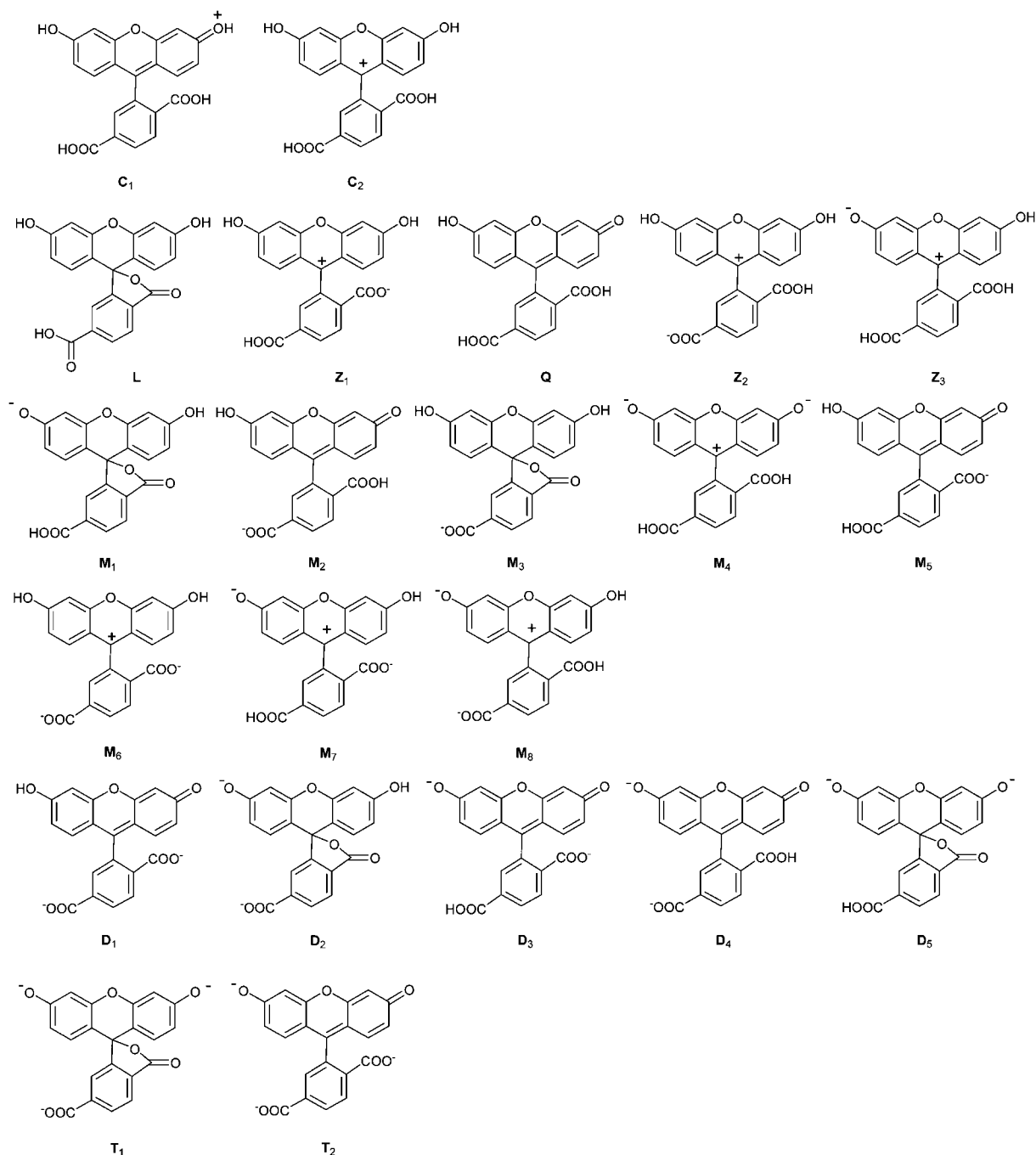(28) Diehl, H.; Horchak-Morris, N. *Talanta* **1987**, *34*, 739–741.

FIGURE 5. Isomers taken into account for the quantum chemical calculation.

a benzene ring. (For comparison, the pK_a of the less acidic carboxy group of terephthalic acid is 4.46.[29])

Nevertheless, it is important to point out that these pK_a values correspond to macroscopic dissociation constants, and it is not the aim of this study to dissect them into the corresponding microscopic counterparts.

The data obtained by chemometric analyses and by quantum-mechanical calculations and, for comparison, the results obtained by the spectrophotometric approach, are reported in Table 1.

The pK_{a2} and pK_{a4} obtained by chemometric analyses are in good agreement within the error with the values obtained from UV–vis determinations. As far as the pK_{a1} is concerned, we

also observe a satisfactory agreement with the chemometric value slightly underestimating the one obtained by UV–vis spectrophotometry. On the other hand, the pK_{a3} values obtained by chemometric analyses and A diagrams are only approximately similar. The difference of more than 1 pH unit of pK_{a3} could be ascribed in this case either to the similar acidity of the ionizable carboxy groups or to the difficulty in isolating the spectrum for the dianion. In any case, Figure 2 points out the good agreement between the spectrophotometric (a) and the chemometric (b) results.

The last comparison in Table 1 was carried out utilizing quantum chemical calculations. In this respect we wish to further remark that the rather crude approximations employed in the PCM do not allow for a quantitative comparison. However, our

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computational results show a relatively good semiquantitative agreement with the experimental data, with the exception of pK$_{a4}$.

Differences with experimental data may be partly due to the crude approximations employed in PCM which may become severely wrong for highly charged species. This limitation may produce an unbalanced and unrealistic thermodynamic description of D $\rightleftharpoons$ T and, consequently a scarce reproduction of pK$_{a4}$.

Conclusions

Ideally, the pK$_a$ of the equilibrium dianion $\rightleftharpoons$ trianion of CF, the trianionic form of CF having the maximum fluorescence among the five prototropic forms, has been found to be 6.7 $\pm$ 0.1 and perfectly matches the normal range of cytoplasmatic pH. A proper measurement of the other three, not previously measured, pK$_a$ values of CF has also been performed. The obtained pK$_a$ values are consistent with previously reported values for fluorescein while the additional pK$_{a3}$ = 4.9 $\pm$ 0.1, very close to that of the less acidic carboxy group of terephthalic acid, is in line with the prevailing dissociation of the second carboxy group on the benzene ring. Quantum chemical calculations, carried out by adopting well tested protocols, provide pK$_{a1}$, pK$_{a2}$, and pK$_{a3}$ values only in qualitative agreement with experimental values. This fact may stem from the approximations adopted in the mean-field approach.

Experimental Section

Materials. CF was purchased from a commercial supplier (purity >99.9%). The buffer solutions[30] employed were (a) HCl−NaCl, covering the pH range 1.0−3.9, (b) CH$_3$COOH−NaOH from pH 3.8 to 5.7, (c) KH$_2$PO$_4$−NaOH, from pH 6.0 to 7.8, and (d) NaOH−NH$_4$Cl, from pH 8.5 to 10.0. All buffer solutions were prepared with the same ionic strength (0.1 M).

Chemometric Analysis. A standard solution was prepared by adding 2 μL of a stock solution of CF in DMSO to 2 mL of buffer solutions. The final concentration of CF was 5.58 $\times$ 10^{-5} M. Each solution was allowed to equilibrate for at least 5 min before the measurement was performed. A spectrum was recorded every 0.02 pH units in the pH interval 1.09–7.69. A final spectrum recorded at pH 10.34 was superimposable to the one recorded at pH 7.69 pointing to the complete deprotonation of CF at the latter pH value.

The aspects which are relevant for the case under study are reported in the Supporting Information. The chemiometric calculations were performed using homemade routines previously tested on literature data.[18,21]

A Diagram Method. A second standard solution was prepared by adding 2 μL of a stock solution of CF in THF to 2 mL of buffer solutions. The final concentration of CF was 5.00 $\times$ 10^{-6} M. Forty-six spectra were recorded in the pH interval 1.07–9.74. The spectra above pH 8.50 can be considered superimposable.

Quantum Mechanical Estimation of pK$_a$ Values. The quantum chemical estimation of pK$_a$ of CF was evaluated in a rather standard fashion as concisely reported in the Supporting Information.

The isomers taken into account for the quantum mechanical calculations are reported in Figure 5. Note that, according to the adopted level of theory, some of the structures reported in Figure 5 were not minima or collapsed to the same structure (see Table S1 in the Supporting Information).

All of the calculations were performed using the Gaussian 03[31] and Gamess[32] programs.

Supporting Information Available: Electronic energies, free energy corrections, and solvation energies (in water) calculated for the species reported in Figure 5; optimized structures; absorbances measured at the selected wavelengths as a function of the pH of the solution; aspects which are relevant for the chemometric analysis and the quantum mechanical estimation; plot of the absorption at 491.5 nm against the pH, in the pH interval 5.7–9. This material is available free of charge via the Internet at http://pubs.acs.org.

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