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Macroscopic pK_a Calculations for Fluorescein and Its Derivatives

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Abstract: This study describes the calculation of the microscopic dissociation and tautomerization constants of fluorescein and its derivatives, 2′,7′-dichlorofluorescein (DCF) and 2′,7′-difluorofluorescein (DFF), in an aqueous environment. In vacuo free energies were obtained using complete basis set (CBS) and DFT-based methods, while free energies of solvation were calculated with the CPCM implicit solvation protocol using the UAHF, UAKS, and Pauling radii sets. Our results indicate that the different vacuum protocols give free energy changes upon dissociation within 1 kcal/mol of each other for a given molecule. Therefore, we suggest that the computationally less intensive PBE1PBE/6-311+G(2d,2p)//PBE1PBE/6-31+G(d) model chemistry may reasonably be used in pK_a calculations of larger molecules. The calculations also provided a rigorous test of the implicit solvation models. Relative calculations of dissociation constants gave results in good agreement with experiment; absolute values deviated from experimental data by 1−3 pK_a units. Consistently better results were obtained with the Pauling radii set. The influence of geometry relaxation on going from vacuum to solvent is negligible for pK_{a2} and larger for pK_{a1} but still smaller than the variation due to the radii set. Calculation of tautomerization constants gave more variable results, with none of the solvation methods able to reproduce experimental values consistently, although certain individual constants were correctly calculated.

Introduction

Proton-transfer reactions in an aqueous environment are of great importance in many areas of chemistry and biology,[1] and a knowledge of the dissociation constant, K_a—more frequently expressed in terms of pK_a—allows for the prediction of the protonation states of a molecule of interest at any given pH value. Consequently, the theoretical prediction of pK_a values using quantum chemical methods has attracted a great deal of interest.[2−20] The various approaches used previously differ in their description of the thermodynamic cycle, the level of molecular orbital theory used for obtaining the energies in a vacuum, and in the methods used to treat solvation effects. The choice of the thermodynamic cycle will not be addressed here, as it has recently been discussed in detail elsewhere.[3,21] However, the level of theory used for vacuum and solvation calculations not only greatly influ-

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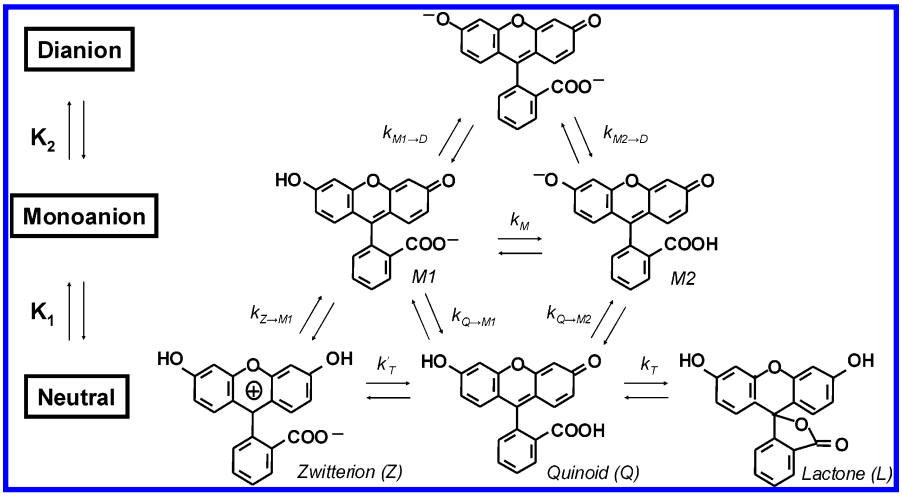


Figure 1. Prototropic equilibria for fluorescein. Tautomerization (k'_T, k_T, k_M) and macroscopic (K_1 and K_2) and microscopic ($k_{Z\rightarrow M1}$, $k_{Q\rightarrow M1}$, $k_{Q\rightarrow M2}$, $k_{M1\rightarrow D}$, $k_{M2\rightarrow D}$) dissociation constants are shown.

ences the accuracy of the results but also the computational tractability and must, therefore, be optimized for a given set of molecules of interest.

It has been reported that pK_a calculations are approaching chemical accuracy for certain classes of organic molecules.[5,22] However, previous studies have tended to focus on predicting dissociation constants for small compounds containing only one ionizable group. While efforts are underway to extend the methodology for larger solutes,[23] the calculation of solvation free energies of doubly charged systems presents significant computational challenges, as indicated by a recent study of acetate and bicarbonate dianions.[24] An assessment of how methods developed for pK_a calculations of small molecules can deal with larger organic species existing in several tautomeric forms and containing multiple ionizable groups is, therefore, of great interest.

Fluorescein and its derivatives, 2′,7′-dichlorofluorescein (DCF) and 2′,7′-difluorofluorescein (DFF, Oregon Green 488), are the subjects of the present study. These molecules have been extensively analyzed in our laboratory[25−27] and are well-known fluorescent probes that have seen widespread use in biological and biochemical applications. In particular, they have been used in fluorescent protein labeling and imaging,[28,29] as Ca^{2+} and pH indicators,[30] and in the measurement of oxidative stress.[25,31,32] Despite the large quantities of experimental data available, there is still a need for more detailed information about the basic chemical properties of these fluorescein-derived probes. The ability to calculate prototropic properties of these dyes is of use both in understanding the probe chemistry and in developing new probes.

Fluorescein, DCF, and DFF can exist in aqueous solution in a number of prototropic forms: cationic, neutral, monoanionic, and dianionic. It follows that three ground-state macroscopic pK_a's are involved. Furthermore, there are three different tautomers for the neutral species—a quinoid, a zwitterion, and a lactonic form—and a further two for the monoanion, with ionized carboxyl or hydroxyl groups.[33,34] Equilibria between the different tautomers and ionization states are described by tautomerization and microscopic dissociation constants. Possible anionic and neutral forms of fluorescein together with microscopic (k) and macroscopic (K_1 and K_2) equilibrium constants are shown in Figure 1. It should be noted that microscopic dissociation constants are readily obtained from theoretical calculations; however, they cannot be directly compared to the experimentally derived macroscopic constants, unless tautomerization constants are also calculated.

We established a three-species model to calculate the pK_a values of the neutral-monoanion and monoanion-dianion transitions, where the neutral form was represented by a quinoid (Q) and the monoanion by the M_1 tautomer (see the Methods section). However, the dissociation constant of the cation was not calculated, since its pK_a is very low, and so is not in the biological pH range.[35]

Conventionally, complete basis set (CBS) model chemistries[36,37] are the methods of choice to obtain vacuum energies, with CBS-QB3[38] being the reference method used in much of the recent work on pK_a calculations. This protocol yields very good agreement with experimental data. However, it is computationally very demanding, both in terms of CPU time and disk storage, and is not practical for calculating the vacuum free energies of the systems studied here; instead, a modified version of the faster CBS-4M[39] was used. A new, state-of-the-art DFT-based protocol for in vacuo energy calculations has recently been proposed,[40] which produces results with an accuracy comparable to the CBS methods but without the computational overhead. In this paper we will compare both CBS and DFT methods for calculating energies of the molecules in a vacuum.

In principle, solvation effects may be evaluated using either continuum[41,42] or explicit solvation models[43−45] or a combination of the two (cluster-continuum methods).[18,46−49] It has been argued that for the proper description of the hydration inclusion of explicit water molecules around the hydrophilic groups of the solute molecule is mandatory.[13] Indeed, several studies have shown that addition of a few explicit water molecules substantially improved the agreement between calculated and experimental solvation energies and pK_a values.[48−50] However, there is still debate on how many water molecules should be added to the solute[18] although Pliego

and Riveros have proposed a variational principle for finding the number of explicit water molecules, such that the optimum is the number of molecules which minimize the solvation free energy.[47] Furthermore, Kelly and co-workers showed that adding one explicit water molecule significantly improves the accuracy of solvation free energies calculated with their new SM6 implicit solvent model[50] but pointed out that it did not work for other models. Given the above and the fact that the molecules studied in this work are rather large and contain multiple ionizable sites, adding explicit water molecules is nontrivial. Therefore, we assess the performance of the more widely used pure implicit solvent model for the calculation of solvation effects on systems of this type, bearing in mind the known shortcomings of the description.

Many different continuum models have been developed (for a recent review see ref 42), and their accuracy depends in part upon the way in which the cavity containing the solute is generated. Since the values of the atomic radii used to generate the solute cavity have been optimized for different levels of quantum mechanical theory, it is important to choose radii corresponding to the method used. Furthermore, Takano and Houk have recently shown that some radii sets are better suited to the calculation of solvation free energies of charged species than others.[51] Consequently, in this work we examine the influence of several radii sets on the results obtained.

Solvation effects should, in principle, include both the solvation free energy change (defined as the energy change upon moving the molecule from vacuum to the solution phase with a fixed geometry)[52] and the relaxation energy (the difference in energy between the molecule optimized in a vacuum and optimized in solvent).[53] However, most studies often include only the former term, calculated using a vacuum-optimal or solvent-optimal geometry, and neglect the relaxation effect. While this neglect may be justified for small molecules, it may not be true for the molecules tested in this work. Therefore, we discuss the effect of this approximation on the quality of the calculated dissociation constants.

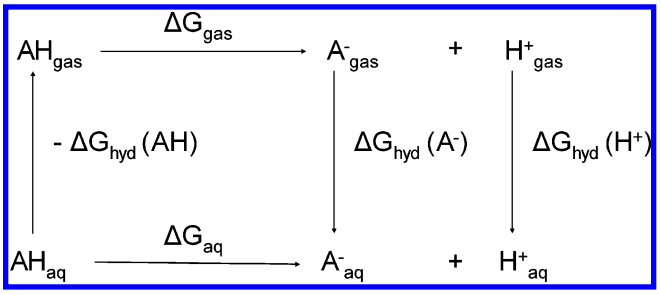
The aim of the present work is to assess the performance of implicit solvent models in the calculation of pK_a values for fluorescein and its derivatives. The protocol should be able to reproduce experimental results to within chemical accuracy but should also be computationally feasible for such large molecules. To this end, we compare results of pK_a calculations for fluorescein and its derivatives using CBS-4M and DFT-based methods for vacuum free energy estimation. Free energies of solvation are estimated by CPCM solvation methods with different radii sets.

Methods

Computational Design. pK_a can be related to the free energy change ΔG_{aq} of the dissociation reaction using the formula

$$pK_a = \frac{\Delta G_{aq}}{RT \ln 10} \quad (1)$$

We have used the following thermodynamic cycle to calculate ΔG_{aq}:



With this cycle

$$\Delta G_{aq} = -\Delta G_{hyd}(AH) + \Delta G_{gas} + \Delta G_{hyd}(A^-) + \Delta G_{hyd}(H^+) \quad (2)$$

and

$$pK_a = \frac{G_{gas}(A^-) - G_{gas}(AH) + \Delta G_{hyd}(A^-) - \Delta G_{hyd}(AH) + G_{gas}(H^+) + \Delta G_{hyd}(H^+)}{RT \ln 10} \quad (3)$$

Here $G_{gas}(H^+) = -6.28$ kcal/mol from the Sackur-Tetrode equation and $\Delta G_{hyd}(H^+) = -264.0$ kcal/mol.[54,55] The value of $\Delta G_{hyd}(H^+)$ was obtained for the 1 atm gas phase/1 M solution standard state and must be converted to 1 M gas phase/1 M solution standard state by adding -1.9 kcal/mol.[56] Note that other values for $\Delta G_{hyd}(H^+)$ have also been suggested. For example, values based on the absolute potential correction for the hydrogen standard electrode range from -254 to -261 kcal/mol.[57] Liptak and Shields used the value of -264.61 kcal/mol derived from the thermodynamic cycle of acetic acid.[4] However, recently the value of -264.0 kcal/mol has been widely accepted as the most reliable estimate of the solvation energy of the proton.[56,58]

When calculating pK_a values of molecules with multiple tautomeric forms, one should take into account all tautomers for a given ionization state. Therefore, both tautomerization and microscopic dissociation constants must be used to calculate macroscopic dissociation constants. From Figure 1 we can define

$$K_1 = \frac{[M][H^+]}{[N]} \quad (4)$$

where [M] is the concentration of all singly ionized tautomers and [N] is the concentration of all neutral tautomers. However,

$$[M] = [M_1] + [M_2] \quad (5)$$

$$[N] = [Z] + [Q] + [L] \quad (6)$$

where M_1, M_2, Z, Q, and L are concentrations of the different tautomers. From Figure 1 we readily obtain expressions for k'_T, k_T, k_M, and $k_{Q\rightarrow M1}$:

$$k'_T = \frac{[Q]}{[Z]} \quad (7)$$

$$k_T = \frac{[L]}{[Q]} \quad (8)$$

$$k_M = \frac{[M_2]}{[M_1]} \quad (9)$$

$$k_{Q\rightarrow M1} = \frac{[M_1][H^+]}{[Q]} \quad (10)$$

Combining all equations, we can derive an expression for K_1 in terms of a microscopic constant $k_{Q\rightarrow M1}$ and tautomerization constants k'_T, k_T, and k_M

$$K_1 = \frac{k_{Q\rightarrow M1} \times k'_T \times (1 + k_M)}{1 + k'_T + (k_T \times k'_T)} \quad (11)$$

and

$$pK_1 = pk_{Q\rightarrow M1} - \log \frac{k'_T \times (1 + k_M)}{1 + k'_T + (k_T \times k'_T)} \quad (12)$$

Similarly,

$$pK_2 = pk_{M1\rightarrow D} + \log (1 + k_M) \quad (13)$$

However, since experimental data for fluorescein and its derivatives show that $[M_2] \sim 0$,[33,59] eqs 12 and 13 may be simplified to

$$pK_1 = pk_{Q\rightarrow M1} - \log \frac{k'_T}{1 + k'_T + (k_T \times k'_T)} \quad (14)$$

$$pK_2 = pk_{M1\rightarrow D} \quad (15)$$

To avoid errors in the absolute pk_a determination we have calculated microscopic pk_as for DCF and DFF relative to the experimental pk_a values for fluorescein. By calculating the difference in pK_a values obtained from eq 3 for two molecules—AH and BH—we obtain the pK_a of AH relative to the pK_a of BH:

$$pK_a(AH) = \frac{1}{RT \ln 10} \times [G_{gas}(A^-) - G_{gas}(B^-) + G_{gas}(BH) - G_{gas}(AH) + \Delta G_{hyd}(A^-) - \Delta G_{hyd}(B^-) + \Delta G_{hyd}(BH) - \Delta G_{hyd}(AH)] + pK_a(BH) \quad (16)$$

In this work AH represented DCF and DFF, and BH represented fluorescein.

The tautomerization constant k_T can be calculated from a free energy change ΔG^T_{aq} of a tautomerization process in solution using

$$k_T = \exp\left(\frac{-\Delta G^T_{aq}}{RT}\right) \quad (17)$$

where ΔG^T_{aq} is the difference in the total free energy in solution between two tautomers.

Gas-Phase Free Energies and Structures. A well-established protocol for calculating free energies in a vacuum is CBS-QB3. However, this proved excessively expensive computationally for the molecules under consideration. Alternative methods were therefore sought, which, on one hand, would be computationally less demanding, and, on the other hand, would still retain the high quality of CBS-QB3. Consequently, we have examined four methods to calculate gas-phase free energies, which are defined below:

V1 − CBS-4M (geometry optimized at HF/6-31+G(d))

V2 − PBE1PBE[60]/aug-cc-pVTZ // PBE1PBE/6-31+G(d)

V3 − PBE1PBE/6-311+G(2d,2p) // PBE1PBE/6-31+G(d)

V4 − PBE1PBE/6-311+G(2d,2p) //CPCM/ HF/6-31+G(d)

In the CBS-4M model chemistry geometry optimization is usually performed at the HF/3-21G level; however, for the molecules studied here, it led to the monoanion accepting a lactonic form, which is not observed experimentally.[61] Since geometries optimized at the HF/6-31+G(d) level maintained a correct, quinoid form, we used this level of theory for CBS-4M energy evaluations.

In the V2 and V3 methods, geometry optimization of a DCF monoanion led to a lactone. In this case, a solvent-optimized geometry, which held the correct quinoid form, was used to perform SP calculations in a vacuum at the PBE1PBE/6-311+G(2d,2p) level. For consistency, we have also performed identical calculations (utilizing solvent-optimized geometries) for fluorescein and DFF and have termed this approach V4. In fact, in this approach all calculations were performed on the solvent geometry; the relaxation effect in going from vacuum to solvent has been neglected.

In all methods, correction for the treatment of internal rotors as harmonic oscillators has been neglected. It should be noted that in principle this might be the source of significant errors if absolute free energy values are of interest.[62] However, the errors would be expected to cancel when calculating relative differences in free energy values for molecules with almost identical internal rotations.

All calculations were performed using the *Gaussian03* software.[63]

Solvation Free Energies and Structures. Free energies of solvation in water were calculated using the CPCM implicit solvent method[64] as implemented in *Gaussian03* with three of the standard radii sets (UAHF,[65] UAKS, and Pauling[66]) using the HF/6-31+G(d) model chemistry, which led to three different solvation energies for each molecule and its corresponding protonation state:

S1 − CPCM(UAHF)/HF/6-31+G(d)

S2 − CPCM(UAKS)/HF/6-31+G(d)

S3 − CPCM(Pauling)/HF/6-31+G(d)

Although the Pauling radii set was originally parametrized for neutral molecules, Takano and Houk[51] have shown that they give the lowest mean absolute deviations (MADs) of solvation energies for all tested radii sets when applied to anions, and so it was of interest to examine whether this extends to dianions. They also showed that the UA0 and UFF radii yield significantly inferior results and so have not been considered here. Furthermore, the UAHF and UAKS radii sets, although closely related, perform quite differently,

especially for anions. Both of these sets are, therefore, included in our study.

The geometries of all molecules were optimized at the HF/6-31+G(d) level and CPCM/HF/6-31+G(d) level using UAHF radii. Relaxation energies were calculated as the difference in SCF energy between solvent- and vacuum-optimized geometries. These were added to the solvation energies to give the total contribution to the energy change upon moving the molecule from a vacuum to a water environment. Note that the relaxation energy can be calculated in a vacuum (as the difference between in vacuo SCF energy for solvent- and vacuum optimized geometries) or in solvent (as the difference between CPCM SCF energy for solvent- and vacuum optimized geometries). Relaxation energies were included in the dissociation constant calculations with methods V1, V2, and V3 but were not used for V4 as this method uses the geometry optimized in water both for calculation of ΔG_{gas} and ΔG_{hydr}.

To investigate the dependence of the optimal geometry in solvent on the radii set, we have performed additional CPCM/HF/6-31+G(d) optimizations using UAKS and Pauling radii on fluorescein. Subsequently, we compared solvation energies calculated for these geometries with energies obtained from single point (SP) calculations using UAKS or Pauling radii on geometries obtained with UAHF radii. Although the absolute values differ by up to 3 kcal/mol, the solvation free energy difference between the pair of molecules (e.g. the neutral species and its anion) is always below 1 kcal/mol (see Supporting Information Table S1). Therefore, the error introduced by not reoptimizing the geometry with different radii sets would be negligible. Consequently, for DCF and DFF, only geometries optimized with UAHF radii are considered.

Tautomerization Constants. To calculate tautomerization constants, the geometries of the neutral zwitterion, quinoid, and lactone forms of fluorescein and its derivatives were optimized at the CPCM/HF/6-31+G(d) level using UAHF radii. Frequency calculations were performed at the same level of theory. The free energy of solvation was calculated using UAHF and Pauling radii sets at the CPCM/HF/6-31+G(d) level, and no relaxation energy was included. G^{T}_{aq} was calculated as the sum of the SCF energy at the PBE1PBE/6-311+G(2d,2p) level, thermal correction to the Gibbs free energy from the CPCM frequency calculations and solvation free energy.

Results and Discussion

Relative pK_a Values. As described in detail in the Methods section, the accuracy of the calculated macroscopic pK_a values depends on two factors: the correct calculation of both the microscopic dissociation constants and tautomerization constants. To dissect the two contributions we have compared calculated microscopic p$k_{Q\rightarrow M1}$ values for fluorescein and its derivatives with the value of p$k_{Q\rightarrow M1}$ obtained from pK_{a1} and tautomerization constants measured experimentally. p$k_{M1\rightarrow D}$ is compared directly to pK_{a2} since $k_M \sim 0$. The comparison of calculated tautomerization constants with experimental values is discussed in a later section.

Table 1. Absolute Values of p$k_{Q\rightarrow M1}$ and p$k_{M1\rightarrow D}$ for DCF Calculated Relative to Fluorescein[a]

	p$k_{Q\rightarrow M1}$		p$k_{M1\rightarrow D}$	
	V1	V4	V1	V4
S1	3.53	4.11	3.18	2.14
S2	3.52	4.09	3.18	2.14
S3	3.80	4.38	4.88	3.84
exp.	3.50		5.19	

[a] Experimental values were obtained from pK_{a1} = 4.00, pK_{a2} = 5.19, k_T = 2.00, k'_T = 7.14, and k_M = 0.[33]

Table 2. Absolute Values of p$k_{Q\rightarrow M1}$ and p$k_{M1\rightarrow D}$ for DFF Calculated Relative to Fluorescein[a]

	p$k_{Q\rightarrow M1}$				p$k_{M1\rightarrow D}$			
	V1	V2	V3	V4	V1	V2	V3	V4
S1	3.37	3.36	3.28	4.49	3.55	3.71	3.75	2.74
S2	3.39	3.38	3.30	4.49	3.55	3.71	3.75	2.74
S3	3.47	3.46	3.37	4.59	4.63	4.80	4.84	3.82
exp.	3.37				4.69			

[a] Experimental values were obtained from pK_{a1} = 3.61 and pK_{a2} = 4.69.[67] k_T = 0.68, k'_T = 16.94 (calculated from the ratio of different tautomers[67]), and k_M = 0.

Tables 1 and 2 show values of p$k_{Q\rightarrow M}$ and pK_{a2} obtained from eq 16. Note that by using eq 16 hydration and vacuum free energies of a proton are not needed, and differences in corresponding free energies, employed in the equation, are likely to lead to the cancellation of errors in solvation and gas-phase free energies.

Analysis of the data shown in Tables 1 and 2 reveals that there is a perfect agreement between the calculated and experimental values for the p$k_{Q\rightarrow M1}$ for all three radii sets and vacuum methods V1−V3. In the case of p$k_{M1\rightarrow D}$ the quality of the results depends on the radii set used. Results obtained with the united atom (UA) radii deviate from experimental data by ~1 (for DFF) and ~2 (for DCF) pK_a units, while the Pauling all-atom radii set reproduces the values correctly. This is surprising since Pauling radii were not optimized for solvation energies of anionic species and should give inferior results compared to the UAHF and UAKS. On the other hand, as Takano and Houk observed, Pauling radii gave the lowest MADs for anionic species from all the radii sets tested. The calculation of p$k_{M1\rightarrow D}$ involves estimation of solvation energies for singly and doubly charged anions, and Pauling radii, according to Takano and Houk's data, are likely to give better estimates of these energies.

It is interesting to note that the protocol V4, which involved calculating vacuum free energies on geometries optimized in water, does not yield correct predictions, with the overestimation of the first dissociation constants and underestimation of the second. This is most likely caused by the incorrect description of the proton dissociation in a vacuum, if the solvent-optimized geometries are used for this process, and by the neglect of the relaxation energy.

Absolute pK_a Values. Given the encouraging results obtained in the relative pK_a calculations, we further estimate microscopic dissociation constants for all molecules using eq 3. This is much more demanding because the absolute values of solvation and gas-phase free energies must be

Table 3. Absolute Values of p$k_{Q\rightarrow M1}$ and p$k_{M1\rightarrow D}$ for Fluorescein[a]

	p$k_{Q\rightarrow M1}$				p$k_{M1\rightarrow D}$ (exp. 6.80)			
	V1	V2	V3	V4	V1	V2	V3	V4
S1	5.08	5.43	5.17	5.94	10.65	10.09	9.62	10.01
S2	5.44	5.80	5.54	6.33	10.65	10.09	9.62	10.01
S3	2.12	2.46	2.21	2.98	8.40	7.85	7.37	7.76
exp.	3.49				6.80			

[a] Experimental values were obtained from pK_{a1} = 4.45, pK_{a2} = 6.80, k_T = 6.09, K'_T = 0.5, and k_M=0.[33]

Table 4. Absolute Values of p$k_{Q\rightarrow M1}$ and p$k_{M1\rightarrow D}$ for DCF[a]

	p$k_{Q\rightarrow M1}$		p$k_{M1\rightarrow D}$	
	V1	V4	V1	V4
S1	5.11	6.56	7.16	5.47
S2	5.49	6.94	7.16	5.47
S3	2.42	3.87	6.61	4.92
exp.	3.50		5.19	

[a] Experimental values were obtained from pK_{a1} = 4.00, pK_{a2} = 5.19, k_T = 2.00, K'_T = 7.14, and k_M = 0.[33]

accurately calculated, and the correct value of the proton solvation free energy must be obtained from experimental data. The latter value is critical for the accuracy of absolute pK_a estimates. As discussed briefly in the Methods section, several different values of the proton solvation energy were used in the past. However, currently, a value of −269 kcal/mol is accepted as the most reliable estimate of the proton solvation energy.[56,58]

Tables 3−5 show the results of the p$k_{Q\rightarrow M1}$ and p$k_{M1\rightarrow D}$ calculations, together with the corresponding experimental data, for fluorescein, DCF, and DFF, respectively. Solvation and vacuum free energies calculated with the methods S1−S3 and V1−V4 are given in the Supporting Information Tables S2−S4 for fluorescein, DCF, and DFF, respectively. For DCF results obtained from the V1 and V4 methods only are shown.

For a given solvation method the differences in the pk_a values produced by the V1−V3 vacuum methods are all below 0.5 pK_a units for the first dissociation constant and below one pK_a unit for the second dissociation constant. All three methods gave convergent results; therefore, the computationally costly methods V1 and V2 may be successfully substituted by the substantially less demanding V3 method. This was further confirmed by the test calculations performed on 3-chloro-4-hydroxyphenol molecule, which showed that methods V1−V3 yield very similar results for $\Delta(G(A^-_{gas})-G(AH_{gas}))$ compared to CBS-QB3 but with a fraction of the computational cost (see Supporting Information Table S5).

As with the relative pK_a calculations, the V4 method does not produce satisfactory results and should be avoided. Good performance of the method in a few cases (such as for the Pauling radii for fluorescein and p$k_{M1\rightarrow D}$ for DCF and DFF) is most probably due to a fortuitous cancellation of errors.

pk_a values presented in Tables 3−5 for the first dissociation constant differ by ∼2 pK_a units for UA radii sets and by ∼1 pK_a unit for Pauling radii. Mean deviations (MDs), given in Table 6, show that the deviation from experimental values for p$k_{Q\rightarrow M1}$ is very similar for all three molecules and indicate

Table 5. Absolute Values of p$k_{Q\rightarrow M1}$ and p$k_{M1\rightarrow D}$ for DFF[a]

	p$k_{Q\rightarrow M1}$ (exp. 3.37)				p$k_{M1\rightarrow D}$ (exp. 4.69)			
	V1	V2	V3	V4	V1	V2	V3	V4
S1	4.95	5.30	4.96	6.94	7.52	7.12	6.69	6.06
S2	5.35	5.69	5.35	7.34	7.52	7.12	6.69	6.06
S3	2.09	2.43	2.09	4.08	6.37	5.97	5.53	4.90
exp.	3.37				4.69			

[a] Experimental values were obtained from pK_{a1} = 3.61 and pK_{a2} = 4.69.[67] k_T = 0.68 and K'_T = 16.94 were calculated from the ratio of different tautomers,[67] and k_M = 0.

Table 6. Mean Deviations (MDs) in pK_a Units of the p$k_{Q\rightarrow M1}$ and p$k_{M1\rightarrow D}$ with Respect to Experimental Values for Different Solvation Methods and Molecules[a]

	fluorescein		DCF		DFF	
	p$k_{Q\rightarrow M1}$	p$k_{M1\rightarrow D}$	p$k_{Q\rightarrow M1}$	p$k_{M1\rightarrow D}$	p$k_{Q\rightarrow M1}$	p$k_{M1\rightarrow D}$
S1	1.73	3.32	1.61	1.97	1.70	2.43
S2	2.11	3.32	1.99	1.97	2.09	2.43
S3	−1.23	1.07	−1.08	1.42	−1.17	1.26

[a] Methods S1−S3 employ the UAHF, UAKS, and Pauling radii sets, respectively. MDs for fluorescein and DFF include pk_a values calculated for the V1−V3 vacuum methods; MDs for DCF are for pk_a values calculated for V1 only.

that there is a systematic error in the calculation of either absolute solvation energies or gas-phase acidities. Indeed, this is the reason why relative p$k_{Q\rightarrow M1}$ values were in such good agreement with experiment. However, as different vacuum protocols yield very similar pk_a values, it must be the solvation energies which deviate from the correct ones and incur the error. This was to be expected, as the correct calculation of solvation free energies for anions would require adding explicit solvent molecules. Bearing this in mind, implicit solvent models are still capable of obtaining p$k_{Q\rightarrow M1}$ values which are within 1 pK_a unit of experimental values for fluorescein-like molecules, if the Pauling radii set is used. It should be noted that UA radii sets cause overestimation of the p$k_{Q\rightarrow M1}$ values, while the Pauling radii set underestimates them.

In the case of the second dissociation constant, the MDs for the values obtained with the UA radii sets differ among test molecules, which indicates that the implicit solvent model is unable to obtain the correct values of solvation energies but, more importantly, is not able to treat the molecules in a consistent way. The difference between calculated and experimental values of p$k_{M1\rightarrow D}$ differ from ∼3 pK_a units for fluorescein to ∼2 pK_a units for DCF. On the other hand, if the Pauling radii set is used, the MDs are similar for all three molecules with an average value of ∼1.2 pK_a units. Again, the fact that the Pauling radii perform better, both in terms of MDs and the ability to sustain a similar level of error, is somewhat surprising given the fact that the Pauling radii set was not optimized for the ionized molecules. On the other hand, UA radii, although optimized on ions, were shown to perform well only if used for the molecules present in the training set.[48]

It should be noted that we have taken reference experimental data from papers by Mchedlov-Petrossyan[33,59] and co-workers (for fluorescein and DCF) and by Orte et al.[67] (for DFF), but other values can be found in the literature.

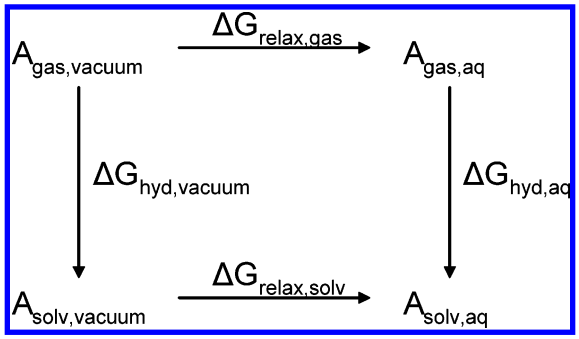


Figure 2. Two ways of solvating a molecule A. The first suffix (gas, solv) describes the environment of the molecule; the second suffix describes the geometry of the molecule (vacuum, aq), e.g. $A_{gas,aq}$ is the molecule in a solvent-optimized geometry being placed in a vacuum. ΔG_{hyd} is the change in free energy upon solvating the molecule, and ΔG_{relax} is the change in free energy upon changing the geometry. Note that generally $\Delta G_{hyd,vacuum} \neq \Delta G_{hyd,aq}$ and hence $\Delta G_{relax,gas} \neq \Delta G_{relax,solv}$.

For example, different authors measured pK_{a1} of fluorescein in the range 4.23−4.39 and pK_{a2} = 6.31−6.72. [34,35,68] Klonis and Sawyer also obtained slightly different tautomerization constants (k_T = 4.3−6.6, k'_T = 0.7−1.1 depending on the dissociation reaction model). For DCF, Leonhardt et al. established pK_{a1} = 3.5 and pK_{a2} = 4.95.[69] Although there is still some disparity within the experimental data, it is much smaller than the error associated with the calculations of dissociation and tautomerization constants, and its impact on the performance of the methods presented in this work is, therefore, minor.

The Influence of Relaxation Energy on the Calculated Dissociation Constants. There is an ongoing debate in the literature on the use of vacuum- or solvent-optimized geometries for the calculation of solvation free energies used in the calculations of dissociation constants. Some investigators have shown that geometry optimization in an aqueous environment is crucial for the correct prediction of pK_a values in cases where substantial charge delocalization is possible, e.g. in aromatic systems.[4,5] Others, however, have not observed such a dependence,[10,24] and geometry optimization in water did not alter the results; in one case it actually made them worse,[11] even though charge delocalization was possible.

To understand fully the significance of the geometry used for the calculation of solvation free energies for pK_a calculations, we should consider the energy difference between the molecule in a vacuum and in solvent, optimized on their respective energy surfaces. As can be seen in Figure 2, this difference comprises two components: the energy change on solvating the molecule (ΔG_{hyd}) and the energy change on going from the vacuum to the solvent geometry (ΔG_{relax}). If ΔG_{relax} is not included, using ΔG_{hyd} calculated on the vacuum-optimized geometry leads to the total free energy of a solvated molecule $G(A_{solv,vacuum})$ being higher than the correct free energy of the molecule A in solvent. This happens because one only takes into account solvation of the vacuum geometry (in terms of $\Delta G_{hyd,vacuum}$) but does not allow the molecule to relax in water, which would lower the total free energy of the system. Conversely, using $\Delta G_{hyd,aq}$ leads to $G(A_{solv,aq})$ being too low. In this case $\Delta G_{relax,gas}$ is neglected. Since this change is made in vacuo the neglected term is always unfavorable. Therefore, it does not matter which geometry is used for the calculation of ΔG_{hyd}, provided that the corresponding correction for the geometry relaxation ΔG_{relax} is taken into account as well. To assess how important the relaxation effect is for our test molecules, we have calculated absolute values of $\Delta G_{relax,gas}$ and $\Delta G_{relax,solv}$ with HF/6-31+G(d) and the Pauling radii set (data shown in Table 7, relaxation energy values for all radii used are shown in Supporting Information Tables S6−S8). The net effect of the relaxation energies on the values of dissociation constants is calculated according to eq 3 as $\Delta pk_{Q\rightarrow M1} = \Delta(M-N)/RT \ln 10$ and $\Delta pk_{M1\rightarrow D} = \Delta(D-M)/RT \ln 10$, where N, M, and D are the relaxation energy values of neutral, mono-, and dianions of respective molecules.

Table 7. Absolute Values of $\Delta G_{relax,gas}$ and $\Delta G_{relax,solv}$ for Fluorescein, DCF, and DFF Molecules [kcal/mol][a]

		neutral	mono-anion	dianion	$\Delta pk_{Q\rightarrow M1}$	$\Delta pk_{M1\rightarrow D}$
fluoroscein	$\Delta G_{relax,gas}$	1.48	2.18	1.75	0.51	−0.31
	$\Delta G_{relax,solv}$	−1.36	−3.10	−3.13	−1.28	−0.02
DCF	$\Delta G_{relax,gas}$	1.34	2.32	1.58	0.72	−0.54
	$\Delta G_{relax,solv}$	−1.23	−2.86	−2.81	−0.77	0.02
DFF	$\Delta G_{relax,gas}$	1.50	2.39	1.78	0.65	0.23
	$\Delta G_{relax,solv}$	−1.36	−3.07	−3.04	−1.23	0.02

[a] $\Delta pk_{Q\rightarrow M1}$ and $\Delta pk_{M1\rightarrow D}$ show the error in a respective pk_a due to the neglect of relaxation.

Analysis of the data in Table 7 reveals that although absolute values of relaxation energies are up to 3 kcal/mol, the net effect of the relaxation energy on the values of dissociation constants is smaller due to the equivalent magnitude of relaxation effects for the pair of molecules (e.g. the neutral species and its anion). Overall, the neglect of relaxation incurs a larger error for the first dissociation constant. If the solvation free energy was calculated on the vacuum-optimized geometry, not including the relaxation energy would lead to the value of $pk_{Q\rightarrow M1}$ being too high by over 1 pK_a unit for fluorescein and DFF. This error would appear to be smaller if the solvation free energy was calculated on the solvent-optimized geometry. On the other hand, for $pk_{M1\rightarrow D}$, the neglect of the relaxation causes only minor errors not exceeding 0.5 pK_a unit. The fact that the relaxation effect is more profound for the first dissociation constant may be explained on the grounds that the solvent has a different influence on the geometry of the neutral and charged molecules; this results in different relaxation energies, which do not fully compensate.

Even though the errors incurred by the neglect of relaxation are on average smaller than errors caused by the inaccurate description of solvent effects, we should be aware that the use of the geometry optimized in a vacuum for the calculation of ΔG_{hyd} will yield higher pK_a values, while the use of geometry optimized in solvent will lead to lower pK_a values. This is, indeed, in line with the findings of earlier studies.[5]

Tautomerization Constants for Fluorescein and Its Derivatives. Tautomerization constants calculated using

Table 8. Experimental[33,67] and Calculated Tautomerization Constants and the Correction to the pK_{a1} (Corr, in pK_a Units) Given by log ($K'_T \times (1 + k_M)/1 + K'_T + (k_T \times K'_T)$)

	fluorescein			DCF			DFF		
constant	UAHF	Pauling	exp.	UAHF	Pauling	exp.	UAHF	Pauling	exp.
k_T	7144	5.86	6.09	452	2.14	2.00	133	0.13	0.68
K'_T	0.18	5E−05	0.50	794	0.007	7.14	2468	0.06	16.94
Corr	−3.85	−4.46	−0.96	−2.66	−2.14	−0.50	−2.13	−1.26	−0.24

different radii sets are shown in Table 8, together with experimental data. Total free energies of all tautomers calculated with both UAHF and Pauling radii are given in the Supporting Information Table S9. We have used only two different radii sets (UAHF and Pauling), given the difference they produced for the pk_a value calculations, and not considered UAKS radii, as they gave solvation energies close to those derived with UAHF.

Experimental data show that the free energy differences between tautomers in aqueous solution are within 1 kcal/mol for most cases, which sets up a challenging task for the computational methods to reproduce the differences correctly: the accuracy of free energy estimates both in vacuo and in solvent must be within 1 kcal/mol.

First, it should be noted that neither of the two radii sets used to obtain free energies is able to predict both tautomerization constants correctly. On the other hand, k_T is very well estimated if the Pauling radii set is used to calculate solvation free energies, although it fails to predict k'_T with reasonable accuracy (errors of 4 orders of magnitude in k or approximately 5 kcal/mol), and for the DCF and DFF molecules, the relative stability of the Q and Z tautomers is reversed. Conversely, UAHF radii are able to reproduce k'_T values for a fluorescein molecule and, within 2 orders of magnitude, for the other molecules and maintain the correct relative stabilities of the Q and Z tautomers. This radii set is also able to describe the correct relative stabilities of the L and Q tautomers of both fluorescein and DCF; however, the errors in k_T are up to 3 orders of magnitude (up to approximately 4 kcal/mol).

Additional contributions to the total free energies of the tautomers in solvent comprise the energy of each tautomer in a vacuum and the thermal correction to the free energy, which includes the zero point energy (ZPE) and entropic contributions. While there are errors also associated with both of these, it has been shown previously that ZPEs converge in the 6-31+G(d) basis set[40] and are, therefore, unlikely to be a significant source of error. To check if the vacuum energy estimation makes a major contribution to the overall error, we have calculated vacuum SCF energies for the fluorescein tautomers using the aug-cc-pVTZ basis set (data not shown), but this has not improved the values of the tautomerization constants. This is also consistent with the pk_a calculations, which show that differences between the V2 and V3 methods are negligible.

As mentioned in the Methods section, tautomerization constants contribute to the values of the macroscopic pK_as; therefore, errors in the former will have a negative impact on the quality of the latter. Table 8 shows the value of the correction to the pK_{a1} value which is due to the existence of different tautomeric forms of the molecule. For all of the molecules under consideration, the fact that the neutral forms of the dyes exist as multiple tautomers increases the pK_{a1} value (see eq 14). This increase is roughly inversely proportional to the stability of the Q tautomer, being the largest for fluorescein and the smallest for DFF and is never larger than 1 pK_a unit. Conversely, corrections calculated with both the UAHF and Pauling radii sets are substantially larger. This is caused by the incorrect prediction of the stabilities of various tautomers and either overstabilization of the zwitterions (Pauling radii) or lactone (UAHF radii). In both cases, the stability of the quinoid form is severely underestimated, leading to an increase in the absolute value of the correction. The use of the calculated tautomerization constants (Table 8) together with microscopic dissociation constants (Tables 3−5) would lead to large discrepancies between calculated and experimental macroscopic dissociation constants, and these discrepancies would be mainly due to the incorrect estimation of tautomerization constants.

Theoretical investigations into the tautomerization constants of fluorescein have been published previously.[70] Those authors used a different solvation method (Poisson−Boltzmann continuum) and were able to reproduce the k_T value correctly but did not calculate k'_T since they failed to obtain an optimized geometry of the zwitterion. Indeed, it has since been shown that implicit solvent models are unable to describe the zwitterionic tautomer of carboxylic monoacids correctly,[15] which may be the reason for the poor prediction of k'_T. It should be noted that the proper calculation of the solvation free energy of the zwitterionic tautomer would require the use of explicit water molecules.

Conclusions

We have used several different calculation protocols to estimate microscopic dissociation and tautomerization constants for fluorescein and two derivatives, DCF and DFF. Evaluation of free energies in a vacuum employed CBS-4M and DFT-based protocols, while solvation free energies were calculated with the CPCM method and several radii sets. We found that all three in vacuo protocols performed similarly. From this we conclude that the least computationally demanding PBE1PBE/6-311+G(2d,2p)//PBE1PBE/6-31+G(d) may be the method of choice for systems which are too large in practice to be treated with the CBS-QB3 model chemistry. Relative calculations of microscopic dissociation constants produced good agreement with experiment for p$k_{Q\rightarrow M1}$ and p$k_{M1\rightarrow D}$ (in the latter case only for Pauling radii). As expected, results of absolute calculations

deviated more from the experimental data with mean deviations between 1 and 3 pK_a units. Better results were obtained if the solvation free energy was obtained with the Pauling radii set.

We have also investigated the contribution of the geometry relaxation on the calculated values of dissociation constants. It turned out that relaxation effects are negligible for p$k_{M1\rightarrow D}$, since geometries of both mono- and dianions are changed similarly, and the net effect is insignificant. For p$k_{Q\rightarrow M1}$ the effect is larger but still smaller than the accuracy of the implict solvent model. In the case of tautomerization constants, the Pauling radii set correctly predicted k_T, whereas UAHF performed better for k'_T; however, neither set gave accurate estimates for both. The results described in this paper indicate that implicit solvent models are able to produce satisfactory results; however, they should be carefully tested for the set of molecules of interest, and appropriate parameters best describing solvation effects should be chosen. This task may be simplified in the future with the development of implicit solvation methods, which are less dependent on a correct choice of parameters to define the solute cavity. Such methods are gradually being developed and include isodensity surface approaches[6,71] or the use of a mixed implict-explicit aqueous environment.[18,47]

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Supporting Information Available: Solvation free energies calculated on geometries optimized with different radii sets (Table S1), solvation and vacuum free energies calculated with the methods S1−S3 and V1−V4 (Tables S2−S4), vacuum free energies of 3-chloro-4-hydroxyphenol (Table S5), relaxation energy values for all radii sets (Tables S6−S8), and total free energies of all tautomers (Table S9). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Bell, R. P. *The Proton in Chemistry*; Chapman and Hall: London, 1973.

(2) Chen, I.-J.; MacKerell, A. D., Jr. *Theor. Chem. Acc.* **2000**, *103*, 483−494.

(3) Liptak, M. D.; Shields, G. C. *Int. J. Quantum Chem.* **2001**, *85*, 727−741.

(4) Liptak, M. D.; Shields, G. C. *J. Am. Chem. Soc.* **2001**, *123*, 7314−7319.

(5) Liptak, M. D.; Gross, K. C.; Seybold, P. G.; Feldgus, S.; Shields, G. C. *J. Am. Chem. Soc.* **2002**, *124*, 6421−6427.

(6) Chipman, D. M. *J. Phys. Chem. A* **2002**, *106*, 7413−7422.

(7) Adam, K. R. *J. Phys. Chem. A* **2002**, *106*, 11963−11972.

(8) Saracino, G. A. A.; Improta, R.; Barone, V. *Chem. Phys. Lett.* **2003**, *373*, 411−415.

(9) Dahlke, E. E.; Cramer, C. J. *J. Phys. Org. Chem.* **2003**, *16*, 336−347.

(10) Magill, A. M.; Yates, B. F. *Aust. J. Chem.* **2004**, *57*, 1205−1210.

(11) Murłowska, K.; Sadlej-Sosnowska, N. *J. Phys. Chem. A* **2004**, *109*, 5590−5595.

(12) Fu, Y.; Liu, L.; Li, R.-Q.; Liu, R.; Guo, Q.-X. *J. Am. Chem. Soc.* **2004**, *126*, 814−822.

(13) Almerindo, G. I.; Tondo, D. W.; Pliego, J. R., Jr. *J. Phys. Chem. A* **2004**, *108*, 166−171.

(14) Busch, M. S. A.; Knapp, E. W. *ChemPhysChem* **2004**, *5*, 1513−1522.

(15) Soriano, E.; Cerdán, S.; Ballesteros, P. *J. Mol. Struct. − Theochem* **2004**, *684*, 121−128

(16) Han, J.; Tao, F.-M. *J. Phys. Chem. A* **2005**, *110*, 257−263.

(17) Han, J.; Deming, R. L.; Tao, F.-M. *J. Phys. Chem. A* **2005**, *109*, 1159−1167.

(18) Kelly, C. P.; Cramer, C. J.; Truhlar, D. G. *J. Phys. Chem. A* **2006**, *110*, 2493−2499.

(19) Namazian, M.; Kalantary-Fotooh, F.; Noorbala, M. R.; Searles, D. J.; Coote, M. L. *J. Mol. Struct. − THEOCHEM* **2006**, *758*, 275−278.

(20) Eckert, F.; Klamt, A. *J. Comput. Chem.* **2006**, *27*, 11−19.

(21) Pliego, J. R., Jr. *Chem. Phys. Lett.* **2003**, *367*, 145−149.

(22) Magill, A. M.; Cavell, K. J.; Yates, B. F. *J. Am. Chem. Soc.* **2004**, *126*, 8717−8724.

(23) Barone, V.; Improta, R.; Rega, N. *Theor. Chem. Acc.* **2004**, *111*, 237−245.

(24) Gao, D.; Svoronos, P.; Wong, P. K.; Maddalena, D.; Hwang, J.; Walker, H. *J. Phys. Chem. A* **2005**, *109*, 10776−10785.

(25) Burkitt, M. J.; Wardman, P. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 329−333.

(26) Wrona, M.; Wardman, P. *Free Radical Biol. Med.* **2005**, *38*, 262−270.

(27) Wrona, M.; Wardman, P. *Free Radical Biol. Med.* **2006**, in press.

(28) Royall, J. A.; Ischiropoulos, H. *Arch. Biochem. Biophys.* **1993**, *302*, 348−355.

(29) Haugland, R. P. *Handbook of Fluorescent Probes and Research products*, 9th ed.; Molecular Probes, Inc.: Eugene, 2002.

(30) Minta, A.; Kao, J. P.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 8171−8178.

(31) LeBel, C. P.; Ischiropoulos, H.; Bondy, S. C. *Chem. Res. Toxicol.* **1992**, *5*, 227−231.

(32) Kane, D. J.; Sarafian, T. A.; Anton, R.; Hahn, H.; Gralla, E. B.; Valentine, J. S.; Ord, T.; Bredesen, D. E. *Science* **1993**, *262*, 1274−1277.

(33) Mchedlov-Petrossyan, N. O.; Rubtsov, M. I.; Lukatskaya, L. L. *Dyes Pigm.* **1992**, *18*, 179−198.

(34) Klonis, N.; Sawyer, W. H. *J. Fluoresc.* **1996**, *6*, 147−157.

(35) Sjöback, R.; Nygren, J.; Kubista, M. *Spectrochim. Acta A* **1995**, *51*, L7−L21

(36) Nyden, M. R.; Petersson, G. A. *J. Chem. Phys.* **1981**, *75*, 1843−1862.

(37) Montgomery, J. A., Jr.; Frisch, M. J.; Ochterski, J. W.; Petersson, G. A. *J. Chem. Phys.* **2000**, *112*, 6532−6542.

(38) Montgomery, J. A., Jr.; Frisch, M. J.; Ochterski, J. W.; Petersson, G. A. *J. Chem. Phys.* **1999**, *110*, 2822−2827.

(39) Ochterski, J. W.; Petersson, G. A.; Montgomery, J. A., Jr. *J. Chem. Phys.* **1996**, *104*, 2598−2619.

(40) Liptak, M. D.; Shields, G. C. *Int. J. Quantum Chem.* **2005**, *105*, 580−587.

(41) Cramer, C. J.; Truhlar, D. G. *Chem. Rev.* **1999**, *99*, 2161−2200.

(42) Tomasi, J.; Mennucci, B.; Cammi, R. *Chem. Rev.* **2005**, *105*, 2999−3093.

(43) Sicinska, D.; Paneth, P.; Truhlar, D. G. *J. Phys. Chem. B* **2001**, *106*, 2708−2713.

(44) Rivelino, R.; Coutinho, K.; Canuto, S. *J. Phys. Chem. B* **2002**, *106*, 12317−12322.

(45) Rode, B. M.; Schwenka, C. F.; Tongraar, A. *J. Mol. Liq.* **2004**, *110*, 105−122

(46) Tuñón, I.; Rinaldi, D.; Ruiz-López, M. F.; Rivail, J. L. *J. Phys. Chem.* **1995**, *99*, 3798−3805.

(47) Pliego, J. R., Jr.; Riveros, J. M. *J. Phys. Chem. A* **2001**, *105*, 7241−7247.

(48) Pliego, J. R., Jr.; Riveros, J. M. *J. Phys. Chem. A* **2002**, *106*, 7434−7439.

(49) Cao, Z.; Lin, M.; Zhang, Q.; Mo, Y. *J. Phys. Chem. A* **2004**, *108*, 4277−4282.

(50) Kelly, C. P.; Cramer, C. J.; Truhlar, D. G. *J. Chem. Theory Comput.* **2005**, *1*, 1133−1152.

(51) Takano, Y.; Houk, K. N. *J. Chem. Theory Comput.* **2005**, *1*, 70−77.

(52) Ben-Naim, A. *Solvation thermodynamics*; Plenum Press: New York, 1987.

(53) Silva, C. O.; Silva, E. C. D.; Nascimento, M. A. C. *J. Phys. Chem. A* **2000**, *104*, 2402−2409.

(54) Tissandier, M. D.; Cowen, K. A.; Feng, W. Y.; Gundlach, E.; Cohen, M. H.; Earhart, A. D.; Coe, J. V.; Tuttle, T. R., Jr. *J. Phys. Chem. A* **1998**, *102*, 7787−7794.

(55) Tuttle, T. R.; Malaxos, S.; Coe, J. V. *J. Phys. Chem. A* **2002**, *106*, 925−932.

(56) Camaioni, D. M.; Schwerdtfeger, C. A. *J. Phys. Chem. A* **2005**, *109*, 10795−10797.

(57) Lim, C.; Bashford, D.; Karplus, M. *J. Phys. Chem.* **1991**, *95*, 5610−5620.

(58) Palascak, M. W.; Shields, G. C. *J. Phys. Chem. A* **2004**, *108*, 3692−3694.

(59) Mchedlov-Petrossyan, N. O.; Kleschchevnikova, V. N. *J. Chem. Soc. Faraday Trans.* **1994**, *90*, 629−640.

(60) Perdew, J. P.; Burke, K.; Ernzerhof, M. *Phys. Rev. Lett.* **1996**, *77*, 3865−3868.

(61) Samoilov, D. V.; Mchedlov-Petrossyan, N. O.; Martynova, V. P.; El'tsov, A. V. *Russ. J. Gen. Chem.* **2000**, *70*, 1259−1271.

(62) Ayala, P. Y.; Schlegel, H. B. *J. Chem. Phys.* **1998**, *108*, 2314−2325.

(63) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; HratchianJ, H. P.; Cross, B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; SalvadorJ, P.; Dannenberg, J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03, Revision C.02 ed.*; Gaussian, Inc.: Wallingford, CT, 2004.

(64) Cossi, M.; Rega, N.; Scalmani, G.; Barone, V. *J. Comput. Chem.* **2003**, *24*, 669−681.

(65) Barone, V.; Cossi, M.; Tomasi, J. *J. Chem. Phys.* **1997**, *107*, 3210−3221.

(66) Lide, D. R. *CRC Handbook of Chemistry and Physics*, 86th edition (June 23, 2005); CRC: 2005.

(67) Orte, A.; Crovetto, L.; Talavera, E. M.; Boens, N.; Alvarez-Pez, J. M. *J. Phys. Chem. A* **2005**, *109*, 734−747.

(68) Smith, S. A.; Pretorius, W. A. *Water SA* **2002**, *28*, 395−402.

(69) Leonhardt, H.; Gordon, L.; Livingston, R. *J. Phys. Chem.* **1971**, *75*, 245−249.

(70) Jang, Y. H.; Hwang, S.; Chung, D. S. *Chem. Lett.* **2001**, *30*, 1316−1317.

(71) Foresman, J. B.; Keith, T. A.; Wiberg, K. B.; Snoonian, J.; Frisch, M. J. *J. Phys. Chem.* **1996**, *100*, 16098−16104.

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