Effects of annulation on absorption and fluorescence characteristics of fluorescein derivatives: a computational study

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Semiempirical molecular orbital calculations (AM1 for structures, INDO/S for electronic excitation energies) for fluorescein and various annulated (linear and angular) derivatives are presented and a strong dependence on the mode of condensation is obtained. While the benzo derivatives 7–9 display a small bathochromic shift only, both the linearly and angularly annullated derivatives 10–18 show a rather pronounced bathochromic shift of absorption and fluorescence in both the neutral and the dissociated forms. The longwave absorptions and emissions make these derivatives useful as dyes for measuring intracellular pH because the spectra are distinctly outside the background emission of cells. The longest wavelength absorptions and emissions are predicted for compounds 10–15. The angularly annulated derivatives 7–12 are found to deviate substantially from planarity. For all anions symmetrical structures are calculated to be more stable than unsymmetrical ones. The solvent effect is treated by the SCRF and also by the supermolecule approximation. Calculated structures as well as electronic transition energies (absorption and emission) for the solvated molecules are completely in line with results obtained for the gas phase.

Introduction

Optical measurement of pH has been known for decades but has not become common practice (except for the use of indicator tests strips) until the specific advantages of optical measurement, in particular of fluorescence-based measurements, were recognised. In recent years, pH indicators have become extremely important for the measurement of intracellular pH,[1] resulting in unique information on pH and its variation inside cells. Moreover, the availability of optical fibres has led to the design of pH sensors for the measurement of pH inside the body and in minute sample volumes.[2,3] Recently, micron size fibre tips have been designed which even allow measurement of pH in single cells.[4]

Fluorescence is the method of choice because of its unsurpassed sensitivity and selectivity. However, one of the disadvantages of fluorescent indicators for the physiological pH range results from the fact that nearly all require short-wavelength excitation, typically in the range from 400 to 550 nm. In this spectral region, the background fluorescence of most real samples (such as blood and cells) is usually very strong. Also, laser light sources operating in this region are expensive. It is highly desirable to use solid-state (semiconductor) components such as light-emitting diodes (LEDs) or laser diodes as light sources, because not only are they small, but they also have minimum power requirements and excellent lifetimes which usually exceed 20 000 h. Numerous efforts have been made to develop fluorescent indicators for the physiological pH range which match the emission lines of superbright LEDs or present day diode lasers.

One of the most widely used pH probes are the fluoresceins.[5] A group of longwave indicators referred to as benzofluoresceins was developed several years ago.[6,7] They have attracted particular interest because they match many of the needs of an ideal pH indicator in that the p*K*s are between 7 and 8, and excitation maxima in the 550 to 650 nm range. In addition (and unlike the fluoresceins), these dyes not only exhibit two absorption bands, but also two emissions (because of a lack of photodissociation in the first excited singlet state), a fact that facilitates ratiometric (two-wavelength) measurement of pH which is of great advantage in cytological studies.[5] In addition, the spectra have clear isosbestic and iso-emissive wavelengths *i.e.* wavelengths where the absorption and emissions, respectively, are pH independent and which therefore may serve as reference wavelengths. As a result, these indicators have found an application in the fluorometric measurement of pH[7] as well as in optical fibre pH sensors[8] and are commercially available now.

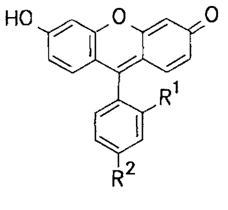
Given the unique properties and the usefulness of these indicators, we have performed a study on these indicator dyes which are derived from fluorescein. The effect of an introduction of a benzo nucleus into the system on the optical spectra strongly depends on the mode of annulation.[6] In order to facilitate the design of dyes with the desired properties described above we have applied computational methods to a series of differently benzo-annulated fluoresceins. Calculational procedures have become increasingly important in the rational design of organic dyes with special properties,[9,10] *e.g.* the development of integrated fluorescent estrogens used for the determination of the estrogen receptor distribution in mammary tumour cells.[11] In addition, the usefulness and reliability of theoretical methods for the description of substituent effects and ionisation on UV–VIS spectra of fluoresceins[12] as well as their solvatochromic properties[13] have already been shown previously. Therefore, we present below calculational results which will be of predictive value.

Results and discussion

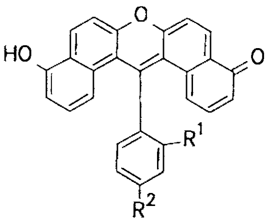
Molecular structures

The spectra of the fluoresceins (**1**–**3**) and seminaphthofluoresceins (**4**–**6**) were taken from the literature (see Tables 1 and 3 for absorption and fluorescence, respectively) while those for compounds **8**, **9**, **17** and **18** were measured in this study. Those of compounds **10**–**15** were only calculated since these molecules do not appear to exist yet.

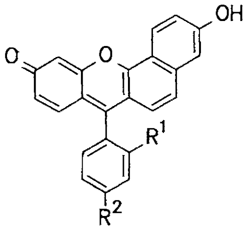
The structures of molecules **1**–**18** were calculated by the semiempirical AM1 method[14] for both the neutral (phenol) as well as the anionic (phenolate) forms. In order to simulate the solvent (experimental investigations as well as the desired applications are done in aqueous buffer) complexes with five water molecules (two in the vicinity of the hydroxy and



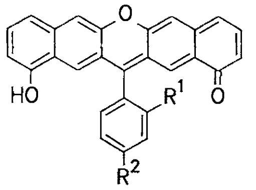
1–3



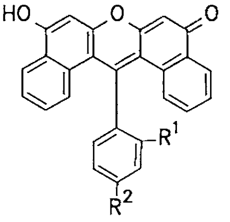
10–12



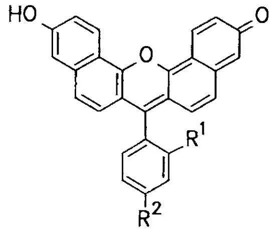
4–6



13–15



7–9

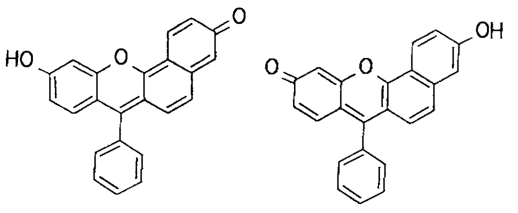


16–18

1, 4, 7, 10, 13, 16 $R^1 = R^2 = H$

2, 5, 8, 11, 14, 17 $R^1 = COOH$, $R^2 = H$

3, 6, 9, 12, 15, 18 $R^1 = H$, $R^2 = COOH$



4A **4B**

Fig. 1 Tautomeric forms of compound **4**

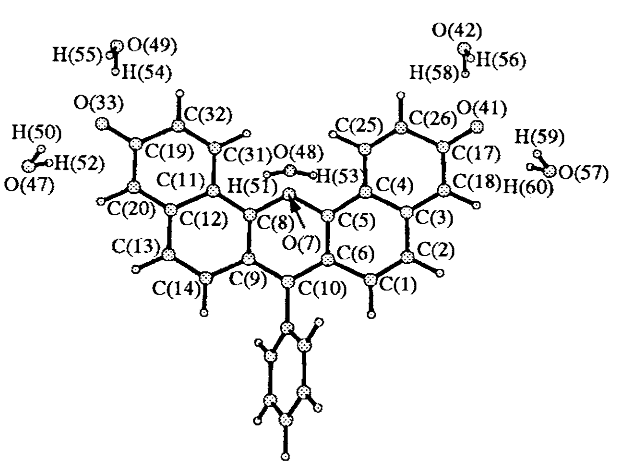


Fig. 2 Calculated (AM1) structure and pertinent interatomic distances (pm; values in parentheses are for the neutral form) of the hydrated anion of compound **16**. r(C1–C2) = 135.4 (136.6), r(C1–C6) = 143.7 (142.6), r(C2–C3) = 144.5 (142.8), r(C3–C4) = 144.4 (141.9), r(C3–C18) = 137.2 (141.4), r(C4–C5) = 139.7 (143.1), r(C4–C25) = 143.4 (142.1), r(C5–C6) = 142.5 (139.5), r(C5–O7) = 138.4 (138.0), r(C6–C10) = 140.2 (144.2), r(C8–O7) = 138.4 (138.3), r(C8–C9) = 142.5 (145.0), r(C8–C11) = 139.7 (137.9), r(C9–C10) = 140.3 (137.4), r(C9–C14) = 143.7 (144.8), r(C11–C12) = 144.4 (145.4), r(C11–C31) = 143.4 (144.3), r(C12–C13) = 144.4 (145.1), r(C12–C20) = 137.2 (136.0), r(C13–C14) = 135.4 (134.8), r(C17–C18) = 144.4 (138.5), r(C17–C26) = 145.9 (142.3), r(C17–O41) = 126.1 (137.4), r(C19–C20) = 144.4 (146.0), r(C19–C32) = 145.9 (146.3), r(C19–O33) = 126.1 (125.3), r(C25–C26) = 135.5 (137.3), r(C31–C32) = 135.5 (134.9), r(O7–H51) = 239.0 (438.6), r(O7–H53) = 239.2 (446.6), r(O33–H50) = 218.1 (221.6), r(O33–H52) = 218.4 (221.8), r(O33–H54) = 218.2 (223.0), r(O33–H55) = 217.5 (222.3), r(O41–H56) = 217.5 (221.6), r(O41–H58) = 218.2 (221.5), r(O41–H59) = 218.0 (364.9), r(O41–H60) = 218.4 (364.3), r(O57–H61) = 209.2 (H61 is only present in the neutral form).

carbonyl oxygen, respectively, and one near the ether bridge) were considered. In agreement with conclusions drawn from UV–VIS spectra[15,16] the (carboxy)phenyl group is calculated to be oriented nearly perpendicular to the plane of the heterocyclic moiety. Except for **7–9** and **10–12** the polycyclic ring moiety of all molecules was calculated to be perfectly planar. Compounds **7–9** and **10–12**, in contrast, are characterised by a strongly distorted ring system. The angle between the planes formed by the two naphthalene halves—as measured by the torsional angle of the lines connecting the centroids of the four benzenoid nuclei—is approximately 40°. The bond to the (carboxy)phenyl substituent bisects this angle. Compounds **4–6**, because they are unsymmetrical, may exist in either one of two tautomeric forms **A** and **B** (see Fig. 1). According to the AM1 calculations—which normally give quite reliable results for tautomeric equilibria[17]—tautomer **B** should be more stable by 42 kJ mol^{-1}. Essentially the same results, even quantitatively, are obtained for the species hydrated by five water molecules or when solvent effects on the tautomeric equilibrium are taken into account by the self consistent reaction field (SCRF) approximation.

For all anions, except of course **4–6**, completely symmetrical structures with equal bond lengths and angles for corresponding atoms, indicating a highly delocalised electron distribution, were obtained. Intermolecular interactions with the solvent as modelled by the hydrated complexes do not disturb this symmetrical electronic structure. In line with this finding is the fact that ionisation of both tautomers **A** and **B** of **4–6** leads to identical anion structures. To illustrate these results, in Fig. 2 the optimised structure, together with some pertinent interatomic distances, of the hydrated anion of **16** is shown. These data clearly show the highly symmetrical structure of the anion as compared to the phenol form as well as the presence of hydrogen bonding between the water molecules and the oxygen atoms contained in the fluoresceins.

However, the solvatochromism of merocyanines has been attributed to a change of the electronic structure from a zwitterionic species in water to a quinoid form in pyridine or chloroform,[18] whereas by theoretical methods invariably only the quinone form was produced.[19] Recently, it has been proposed that the geometry should be optimised in the presence of sparkles (positive or negative point charges) followed by a single point calculation without them to obtain the true energy of the polarised structure.[20] We have adopted an analogous approach to model the possible effect of counterions on the electronic and geometrical structure of the anions. Irrespective of where the sparkle is initially placed (*e.g.* the ether, carboxy or hydroxy oxygen atom), except for **16–18**, geometry optimisation leads to an unsymmetrical structure (phenolate–quinone) with the sparkle moving from the initial position to the vicinity of the phenolate oxygen. Only in the cases of **16–18** does a sparkle placed near the ether oxygen remain there leading to a symmetrical anion. Generally, these unsymmetrical structures are inherently less stable than the symmetrical ones by ≈10 kJ mol^{-1}. Interestingly, for the hydrated species this preference for the symmetrical structures appears to be even more pronounced. Here the energy differences are in the range of 21 kJ mol^{-1} (**4**) to 82 kJ mol^{-1} (**7**).

Table 1 Calculated and experimental excitation energies $v/10^{-3}$ cm^{-1} for the investigated compounds (calculated oscillator strengths in parentheses)

	Phenol-form				Phenolate-form		
Comp.	Gas phase	SCRF	$+5H_2O$	Exp.	Gas phase	$+5H_2O$	Exp.
1	25.3 (0.72)	25.0 (0.87)	25.1 (0.78)		21.2 (0.98)	21.4 (1.00)	
2	25.2 (0.71)	25.0 (0.84)	25.2 (0.77)	22.2[16]	21.2 (1.00)	21.3 (1.01)	20.2[16]
3	25.3 (0.72)	24.8 (0.85)	25.0 (0.77)		20.7 (0.86)	21.1 (0.95)	
4A	21.6 (1.15)	21.9 (1.05)	21.2 (1.16)		19.4 (1.11)	19.2 (1.08)	
4B	24.3 (0.63)	23.7 (0.73)	23.8 (0.62)		19.4 (1.11)	19.2 (1.08)	
5A	21.4 (1.13)	21.4 (1.08)	21.3 (1.14)		18.9 (1.08)	19.2 (1.09)	
5B	24.0 (0.63)	23.8 (0.70)	23.9 (0.63)		18.9 (1.08)	19.2 (1.09)	
6A	21.2 (1.15)	21.0 (1.12)	21.2 (1.16)	18.4[7]	18.8 (1.03)	18.9 (1.07)	
6B	24.0 (0.61)	23.4 (0.70)	23.7 (0.63)	19.8; 20.8[7]	18.8 (1.03)	18.9 (1.07)	18.6[7]
7	24.1 (0.78)	23.4 (0.84)	23.9 (0.84)		19.8 (1.00)	20.3 (0.99)	
8	24.0 (0.77)	23.6 (0.84)	23.8 (0.81)		19.5 (0.96)	19.6 (0.98)	18.7[6]
9	24.0 (0.77)	23.4 (0.82)	23.7 (0.83)		19.4 (0.89)	19.6 (0.95)	18.3[a]
10	18.3 (0.73)	17.5 (0.81)	18.0 (0.73)		15.1 (0.92)	15.1 (0.90)	
11	18.4 (0.74)	17.8 (0.80)	18.2 (0.75)		15.2 (0.92)	15.3 (0.91)	
12	18.3 (0.73)	17.7 (0.80)	18.2 (0.74)		15.1 (0.93)	15.1 (0.92)	
13	18.4 (0.91)	18.2 (0.99)	18.1 (0.90)		15.2 (1.26)	15.1 (1.24)	
14	18.4 (0.90)	19.1 (0.98)	18.2 (0.89)		15.2 (1.27)	15.1 (1.24)	
15	18.4 (0.92)	17.8 (0.76)	18.2 (0.90)		15.0 (1.27)	14.9 (1.25)	
16	20.6 (0.95)	20.8 (0.87)	20.5 (0.98)		17.2 (1.12)	17.3 (1.13)	
17	20.7 (0.94)	20.8 (0.91)	20.5 (0.97)		17.2 (1.14)	17.3 (1.12)	16.5[6]
18	20.5 (0.95)	20.3 (0.96)	20.4 (0.97)	19.5[a]	17.0 (1.11)	17.1 (1.11)	16.7[a]

[a] Experimental values (this work) for compounds with $R^1 = R^2 =$ COOH.

Table 2 Calculated excitation energies $v/10^{-3}$ cm^{-1} and oscillator strengths (in parentheses) for the anions of the investigated compounds

Comp.	Symmetrical	Unsymmetrical[a]	Unsymmetrical[b]
1	21.2 (0.98)	23.0 (0.94)	21.1 (0.98)
1 + $5H_2O$	21.4 (1.00)	22.6 (0.97)	21.2 (0.98)
4	19.4 (1.11)	22.4 (0.89)	19.5 (1.00)
4 + $5H_2O$	19.2 (1.08)	19.9 (0.94)	19.0 (1.02)
7	19.8 (1.00)	21.4 (0.96)	19.8 (0.98)
7 + $5H_2O$	19.8 (1.01)	21.2 (0.97)	19.8 (0.98)
10	15.1 (0.92)	17.2 (0.85)	15.3 (0.81)
10 + $5H_2O$	15.1 (0.90)	16.6 (0.88)	15.3 (0.84)
13	15.2 (1.26)	17.0 (1.14)	15.2 (1.10)
13 + $5H_2O$	15.1 (1.24)	15.2 (1.21)	15.2 (1.19)
16	17.2 (1.12)	19.2 (1.07)	17.3 (1.06)
16 + $5H_2O$	17.3 (1.11)	19.0 (1.07)	17.4 (1.07)

[a] With counterions. [b] Without counterions.

Excitation energies

Based on AM1 geometries, electronic excitation energies were calculated by the INDO/S procedure.[21] For neutral species, in addition to the supermolecule approach, the solvent effect on UV–VIS spectra was also taken into account by the SCRF approximation.[22] The results of these calculations are collected in Table 1.

As is also found experimentally,[15,16] the effect of carboxy groups (R^1 and/or R^2 = COOH *vs.* H) on the calculated excitation energies for both neutral molecules as well as anions is negligible. The solvent effect calculated either by the SCRF method, which cannot describe hydrogen bonding effects, or the supermolecule approximation is rather small and somewhat irregular. Experimentally, a hypsochromic shift in hydrogen bonding solvents is found,[15,23] *e.g.* for the fluorescein dianion a shift of ≈900 cm^{-1} compared with a calculated shift of 200 cm^{-1} is found in going from *N*,*N*-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) to aqueous solution.[23] Generally, the calculated transition energies are somewhat too high (< 3000 cm^{-1}) as compared with the experimental values. However, trends such as the shorter wavelength absorption of tautomer A of **4**–**6** compared with **B**[7] are satisfactorily reproduced by the calculations. The most striking feature, however, is the pronounced effect of the mode of benzo-annulation on the electronic absorption and emission spectra. Condensation of two benzo nuclei onto fluorescein, resulting in compounds **7**–**9**, has a rather small effect which is comparable to fusion of only one ring to give seminaphthofluoresceins **4**–**6** (see Table 1). As a consequence for dibenzofluoresceins **7**–**9** the calculated excitation energies are higher by ≈2000–3000 cm^{-1} than those of naphthofluoresceins **16**–**18**. The deviation from planarity obtained for these angularly fused systems obviously cannot be used to explain this effect since for compounds **10**–**12**, which are also highly distorted, the calculations predict a much larger bathochromic shift. Apparently, the mode of annulation in **7**–**9** only leads to a perturbed fluorescein rather than an enlarged chromophor as in compounds **10**–**18**. Compounds **10**, **13**, **16** (or **11**, **14**, **17** or **12**, **15**, **18**) are structural isomers possibly formed in the reaction of 1,6-dihydroxynaphthalene with the corresponding aromatic carboxylic acid derivative, *e.g.* phthalic anhydride for **11**, **14** or **17**.[6] Interestingly, both angular fusion to give the nonplanar molecules **10**–**12** as well as linear condensation (**13**–**15**) should lead to a considerably longer wavelength absorption by ≈2000 cm^{-1} than the isomeric angular condensation to **16**–**18**. These results hold for calculations in the gas phase as well as in solution.

The results of the effect of the anion structure (symmetrical *vs.* unsymmetrical with and without counterion) on calculated transition energies are collected in Table 2. Generally, in the presence of a counterion the calculations lead to a hypsochromic shift of 1000–2000 cm^{-1}. By itself, *i.e.* in the absence of a counterion, an unsymmetrical structure of the anion is predicted to exert a rather small influence on the absorption energy. In solution, where counterions are free to move, therefore, only a negligible effect on the excitation energies of the anions is to be expected. As in the case of the undissociated forms the anions, independent of whether the symmetrical or unsymmetrical structures are used in the calculations, of dibenzofluoresceins **7**–**9** are predicted to absorb at considerably shorter wavelengths than naphthofluoresceins **16**–**18** and their isomers **10**–**15** by ≈2000 cm^{-1} (see Table 2), in excellent agreement with experimental results for **8** and **17**[6] and the derivatives with $R^1 = R^2$ = COOH. As for the undissociated forms, the longest wavelength absorptions are calculated for **10**–**15**. Finally, in Table 3 the calculated fluorescence spectra of the anions of the investigated compounds (for $R^1 = R^2$ = H) are listed. The above mentioned annullation effect is also clearly visible in the calculated fluorescence spectra of these compounds. Again, the longest wavelength emission is

Table 3 Calculated and experimental fluorescence (10^{-3} cm^{-1}) of the anions

Comp.	Gas phase	$+5\,H_2O$	Exp.
1	19.8	20.2	19.4[6]
4	19.0	17.9	16.0[7]
7	17.7	19.5	17.7[6]
10	14.5	14.3	
13	14.3	14.1	
16	16.3	16.4	15.2[6]

predicted for the two isomers **10** and **13**. With respect to the intended application of these compounds as materials for pH sensors in biological samples compounds **10–18** seem to be the most promising. One of the main conclusions of this paper is that **10–15** should absorb and emit at considerably longer wavelengths than any other isomeric benzo- or naphthofluorescein. Thus, besides compounds **16–18** which already have attracted considerable interest,[5,6] compounds **10–15** appear to be worthwhile targets for synthesis and detailed experimental investigation.

Experimental

Absorption and emission spectra of carboxydibenzofluorescein **7** and carboxynaphthofluorescein **16** ($R^1 = R^2 = COOH$) were measured in methanolic sodium dihydrogen phosphate buffer solutions on a Shimadzu UV-2101-PC photometer and RF-5001-PC fluorophotometer, respectively. Adjustment of pH was done with 2 M aqueous NaOH. In the calculations, all geometries were completely optimised (keyword PRECISE) by the eigenvector following routine using the AM1 method.[14,24,25] Excitation energies were obtained by INDO/S[21,26] using 380 singly excited configurations in the CI expansion. Starting structures for the geometry optimisation by the semiempirical methods were generated with the aid of the SYBYL molecular modelling package.[27] For the hydrated species the water oxygen atoms were placed at a distance of ≈3 Å from the carbonyl, ether and hydroxy groups of the fluoresceins. The hydrogen atoms of the water molecules were oriented towards the oxygens. For the calculation of fluorescence spectra the first excited singlet state was optimised by AM1 with the HOMO–LUMO singly excited configuration as starting determinant (keywords SINGLET ROOT = 2 CI = 2). This optimised geometry was then used in the calculation of transition energies by INDO/S.

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