The Unusually Strong Effect of a 4-Cyano Group upon Electronic Spectra and Dissociation Constants of 3-Substituted 7-Hydroxycoumarins

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Synthesis, absorption and fluorescence spectra as well as pK_a values of 7-hydroxycoumarins with electron-withdrawing substituents in positions 3 are described. Introduction of a 4-cyano group is achieved by oxidative cyanation of coumarins using potassium cyanide and elemental bromine. 7-Hydroxycoumarins without a 4-cyano group are useful indicators for measuring physiological pH values due to their intense fluorescences, longwave absorptions and emissions, as well as pK_a values of around 7. The presence of a 4-cyano group gives rise to a dramatic longwave shift in absorption (30—40 nm in methanol) and emission (55—80 nm). In water solution, the fluorescence maxima are at around 570—600 nm, with excitation maxima between 410 and 510 nm, depending on whether the phenol or phenolate species is excited. For all coumarins under investigation, fluorescence is from the anion form even in the pH 2—7 range. This phenomenon is interpreted in terms of excited state dissociation according to the Förster model. The interpretation is corroborated by calculations of the excited state pK_a values, which show them to be lower by 4.5—6.4 units than those of the ground state.

7-Hydroxycoumarins such as 4-methylumbelliferone (4-MU) play an important role in measuring physiological pH values by fluorimetry.[1-6] In addition, esters, ethers and glycosides of 4-MU are frequently used- and commercially available-enzyme substrates for use in clinical chemistry and enzymology. The formation of fluorescent 4-MU from practically nonfluorescent ethers and esters is a measure for the enzyme activity.[7,8]

However, 4-MU has two decisive disadvantages: (a) Its pK_a value of 7.8 is relatively high, so that under physiological pH conditions only partial dissociation to the highly fluorescent anion (fluorescence maximum 450 nm) takes place;[9] (b) its fluorescence is maximally excited between 330 and 360 nm, a spectral region where background fluorescence from biological matter such as serum or intracellular liquids is fairly high, mostly due to the presence of reduced nicotinamide adenine dinucleotide.

By introducing an electron-withdrawing substituent in position 3, we hoped to shift the excitation and emission maxima to longer wavelengths, and simultaneously to lower the pK_a values. The bathochromic effect of certain substituents in position 3 is known in the chemistry of optical brighteners derived from 7-aminocoumarins.[10]

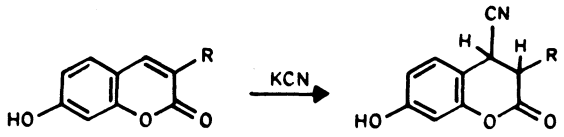
The emission maxima of 3-substituted umbelliferones in alkaline solutions have been reported by Sherman and Robins, and fluorescence intensity was correlated with Hammett σ-constants.[11] More recently, we have reported on the photodissociation of related umbelliferones.[12] Their properties as laser dyes have also been described.[13] The effect of substituents in position 3 on spectra and pK_a values of umbelliferones has not been studied so far.

Results and Discussion

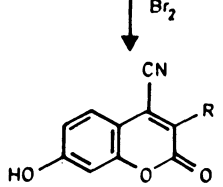
Syntheses. 7-Hydroxycoumarins with a substituent in position 3 (**1a**—**i**) can be prepared according to one of the numerous methods decribed in the literature including patents.[10,14] We found the procedure of condensing 2,4-dihydroxybenzaldehyde with an appropriate CH_2-acidic ester or acid such as ethyl 2-benzoxazolylacetate in the presence of pyridine (as a solvent) and aniline (as a catalyst, 1 mol-equiv), most suitable.[15]

Introduction of a 4-cyano group into umbelliferones was accomplished by addition of potassium cyanide to a suspension of the coumarin in *N,N*-dimethylformamide (DMF) and subsequent dehydrogenation with elemental bromine. We assume that leuko-dyes of general structure **2** are reaction intermediates, which are then oxidized to the respective cyanocoumarins **3a**—**e**.

Oxidative cyanations of dyes with activated double bonds have been described by various authors.[16-20] Oxidizing agents such as ferric ion.[16,19] lead tetra-



1a - i [2]



3a - e

1, 3	R
a	2-Benzoxazolyl
b	2-Benzothiazolyl
c	5-Methyl-7-sulfonato-2-benzoxazolyl, potassium salt
d	5-Chloro-2-benzoxazolyl
e	2-Benzimidazolyl
f	2-Furyl
g	2-Thienyl
h	COOH
i	Phenyl

acetate,[17,18,21] bromine,[21] peroxides, and other oxidizing agents[19] have been proposed.

It should be noted that, for unknown reasons, we failed to prepare the 4-cyano derivatives of compounds **1f—i**. **3c** was synthesized by oxidative cyanation of the potassium phenolate **1c**, since when employing the free 7-hydroxy compound no reaction took place. **3e** could be prepared in low yield only, possibly due to competitive bromination of the imidazolyl NH.

The structures of the cyanoumbelliferones were confirmed by correct analytical and spectral data. The IR spectra are characterized by carbonyl bands in the 1710—1750 cm^{-1} range, and the lack of an intense band of the cyano group. If at all observable, the band consists of a very weak signal at around 2240 cm^{-1}.

The ^{1}H-NMR spectra are of little significance, since most of the protons come to lie in δ=6.7—8.1 range, except for the methyl group present in **3e**. The protons of the 7-hydroxy groups show no sharp signal in pure dimethyl sulfoxide. However, in the presence of traces of water a signal can be observed at around δ=11.2.

The mass spectra consist of strong signals for the molecular ion (M^+) and fragments resulting from the elimination of CN (M^+-26), HCN (M^+-27), and CO (M^+-28). In each case, the molecular ion peak is the 100 % (base) peak.

The dyes are fairly stable in neutral and acidic aqueous solutions, albeit much less than the respective cyanounsubstituted coumarins. They suffer, however, considerable decomposition at pH values above 8. The 4-cyano group is not stable under sulfonation conditions (*e.g.*, oleum). Therefore, sulfonato groups have to be introduced prior to cyanation.

Electronic Spectra. The spectra of 7-hydroxycoumarins **1a—i** in water-free organic solvents consist of an intense absorption band in the 365—390 nm range, and an emission maximum between 450 and 470 nm (Table 1), except for compounds **1h** and **1i**, which absorb distinctly shorter (*ca.* 340 nm).

In aqueous solution both the absorption and fluorescence spectra are highly pH-dependent in the near neutral pH range, a fact that prevents the use of 7-hydroxycoumarins as optical brighteners.[10] The absorption and fluorescence data of aqueous pH 4 and pH 9 solutions are summarized in Table 1. While the fluorescence maxima do not change significantly

Table 1. Longest-wave absorption and fluorescence maxima of 7-hydroxycoumarins **1** and **3** in various solvents[a]

Compound	Solvent[a]	Absorption maximum nm	ε M^{-1} cm^{-1}	Fluorescence maximum nm	Compound	Solvent[a]	Absorption maximum nm	ε M^{-1} cm^{-1}	Fluorescence maximum nm
1a	A	370	—	458	**1h**	A	336	—	403
	B	377	31.800	448		B	337	15.300	408
	C	377	28.200	469, 435(sh)		C	342	—	447, 405(sh)
	D	427	44.300	471[c]		D	386	—	448[c]
1b	A	378	—	462	**1i**	A	336	—	422
	B	390	29.800	465		B	342	23.000	430
	C	385	31.000	485, 465(sh)		C	338	19.700	465, 428
	D	439	47.000	490[c]		D	383	26.100	462[c]
1c	A	—[b]	—	—	**3a**	A	405	—	517
	B	380	34.400	451		B	409	25.600	516
	C	378	32.000	468, 435(sh)		C	416	24.100	575, 520(sh)
	D	431	44.000	470[c]		D	494	33.200	577[c]
1d	A	372	—	456	**3b**	A	420	—	518
	B	378	30.600	450		B	430	28.300	520
	C	378	—	470, 440(sh)		C	432	23.300	594, 520(sh)
	D	425	—	472[c]		D	505	33.100	595[c]
1e	A	—[b]	—	—	**3c**	A	—[b]	—	—
	B	387	33.000	466		B	408	23.000	519
	C	385	—	477, 435(sh)		C	416	—	574, 510(sh)
	D	427	—	479[c]		D	494	—	577[c]
1f	A	370	—	430	**3d**	A	408	—	509
	B	370	26.300	454		B	411	27.100	519
	C	368	—	468, 435(sh)		C	418	21.400	576
	D	405	—	489[c]		D	497	32.400	577[c]
1g	A	367	—	448	**3e**	A	419	—	509
	B	369	25.000	466		B	418	25.400	528[d]
	C	365	—	476, 455(sh)		C	419	—	590, 530(sh)
	D	407	—	500[c]		D	487	—	593

a) A, benzene; B, methanol; C, buffer of pH 4 (citrate); D, buffer of pH 9 (borate). b) Insoluble. c) Very intense. d) In acetone.

with pH between pH 4 and 9, the relative intensities are around two times higher in alkaline solutions than in acidic solution.

The presence of a 4-cyano group results in a considerable bathochromic shift in both absorption and emission. All cyanocoumarins are orange-red (absorption maxima 405—430 nm) and exhibit bright green fluorescence in organic solvents. The maxima suffer a longwave shift with increasing solvent polarity. The molar absorbances are somewhat lower than those of compounds **1**. The spectra of 4-cyanoumbelliferones in alkaline solutions are far red-shifted in both absorption and emission, when compared with the respective phenol species. In addition, they exhibit large Stokes shifts of 80—90 nm, which, however, in terms of wavenumbers is about the same as for coumarins **1a—i** (3000—4500 cm^{-1}).

pH Effects. The pK_a values of selected umbelliferones were determined by spectrophotometry and are listed in Table 2. As can be seen, the values are temperature dependent and decrease by 0.012—0.016 units per °C increase in temperature. Coumarins possessing a 4-cyano group display distinctly increased acidities (0.6—0.8 units lower).

It is noted that fluorescence of 7-hydroxycoumarins **1** and **3** is almost invariably from the anion form in the pH 4—9 range, despite of photoexciting the undissociated* species in acidic solutions. According to Förster,[22] this can be interpreted in terms of photodissociation due to a decrease in pK_a in the first excited singlet state (S_1-state).

We have calculated the pK_a values of the S_1-state with the help of the following equation,[22-24]

$$pK_a(S_1) = pK_a(S_0) - \frac{N \cdot h}{2.303RT}(\nu'_{BH} - \nu'_B)$$

wherein ν'_{BH} and ν'_B are the wavenumbers of the O–O transition of phenol and phenolate, respectively. The mean of the wavenumbers of absorption and fluorescence was taken as O–O transition.[23]

The results are given in Table 2. They show all coumarins to become stronger acids by 4.5—6.4 pK_a units, a fact that can explain the appearance of anion fluorescence even in weakly acidic solutions: When photoexciting a coumarin such as compound **1a** in pH 4 solution, it will dissociate during the lifetime of the S_1-state by virtue of its $pK_a(S_1)$ of 1.74 (Table 2).

It is, however, noted that shortwave shoulders are in most cases observed in the fluorescence emission bands of coumarins **1** and **3** in pH 4 solutions. These shoulders are attributed to emissions from the neutral species, occurring prior to the S_1-state dissociation step.

Concluding Remarks. We have presented here a series of fluorescent compounds with interesting spectral properties. Their pK_a values between 6 and 7 may render them useful longwave excitable and fluorescing indicators for measuring physiological pH values. Owing to their lower pK_a values, cyanocoumarins **3** are more suitable to measure pH's of weakly acidic bioliquids such as urine, whereas coumarins **1** are more useful to measure near-neutral pH's (such as in blood).

Esters of coumarins **1** and **3** with carboxylic, phosphoric and sulfuric acids, respectively, as well as ethers and glycosides are useful enzyme substrates for direct and continuous kinetic determination of hydrolyses.[25] These results will be reported elsewhere.

Experimental

Melting points are uncorrected. Spectra were recorded on the following instruments: Perkin Elmer Lambda 5 (UV/VIS); Aminco SPF 500 (fluorescence); Perkin Elmer 421 (IR); Varian A60 A (^{1}H-nmr); Varian-Mat 111 (mass spectra). Elemental analyses were performed on a Carlo-

Table 2. Ground state and first excited singlet state pK_a values of umbelliferones **1** and **3** at 22 °C

Compound	Ground state pK_a value	O-O-transition/cm^{-1} a)		Calculated Excited state pK_a	ΔpK_a
		Phenol species	Phenolate species		
1a	6.84±0.04	24.757	22.325	1.74	−5.10
1b	7.02±0.03; 6.79b)	23.740	21.594	2.50	−4.51
1c	6.80±0.08; 6.60b)	24.722	22.239	1.59	−5.21
1d	6.80±0.07	24.591	21.186	2.11	−4.69
1e	7.01b)	24.481	22.148	2.11b)	−4.90
1f	7.41b)	25.081	22.570	2.14b)	−5.27
1g	7.71±0.05	24.688	22.285	2.67	−5.04
1h	7.04±0.02	26.965	24.114	1.06	−5.98
1i	7.80±0.05	26.475	23.877	2.35	−5.45
3a	6.07±0.04	21.708	18.787	−0.06	−6.13
3b	6.38±0.03	21.190	18.304	0.32	−6.06
3c	6.08±0.04	21.823	18.787	−0.29	−6.37
3d	6.00±0.04	21.614	18.726	−0.06	−6.06

a) The mean of the longest-wave absorption and fluorescence maximum (in cm^{-1}) was taken as O-O-transition; the wavelength of the shortwave shoulder in pH 4 solution, which is attributed to emission from the neutral form, was used for the calculation of the values for the phenol species. b) At 37 °C.

* The expression "undissociated" refers to the phenol group, not to the sulfonato group.

Table 3. Physicochemical properties as well as spectral data for coumarins **3a—e**

Compound	Mp θ_m/°C (cryst. from)	Yield/%	IR (cm^{-1})	^{1}H-NMR (in DMSO-d_6) (δ-units, TMS=0)	Mass spectrum
3a	282—285 (Ethanol)	49	3300, 1750, 1620 1590	6.70—7.90 (m, 7H)	—
3b	336—338 (Dec.) (Ethanol)	61	3330, 1730, 1700, 1620, 1590	6.80—8.10 (m, 7H) 11.2 (s, 1H)	320 (M^+, 100%), 292 (27%)
3c	>350 (Ethanol/water, 1:1)	63	3400, 1730, 1620, 1590	2.35 (s, 3H), 6.80—7.80 (m, 5H), 11.3 (br.s, 1H)	—
3d	291—294 (Ethanol)	52	3400, 1745, 1620, 1590	6.70—7.90 (m, 6H)	338 (M^+, 100%), 310 (37%)
3e	322 (dec.) (Ethanol)	21	3340, 1710, 1620, 1555	—	303 (M^+, 100%) 275 (21%)

Erba 1106 C,H,N-analyzer. pH values were measured with a pH electrode (Metrohm, Switz.) that was calibrated against pH 7 and pH 4 standard buffers.

General Procedure for the Preparation of 4-Cyano-7-hydroxycoumarins ***3a—e.*** 2.6 g (0.04 Mol) potassium cyanide, dissolved in 6 ml water, are added at room temperature to a suspension of 0.02 mol of the respective 7-hydroxycoumarin (**1a—e**) in 50 ml *N,N*-dimethylformamide. Note that **1c** should be employed as its potassium phenoxide salt. The mixture is stirred for 1 h at 40 °C. After filtration, 1 ml (0.02 mol) elemental bromine is added dropwise to the filtrate at 0 °C. The mixture is then stirred for 1 h at this temperature. The resulting precipitate is collected, washed with water and methanol and purified by double recrystallization from the solvent given in Table 3. Physicochemical and spectral data of the new compounds are given in Table 3. All compounds gave C,H, and N-analyses in agreement (deviation maximally ±0.3 %) with calculated values.

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