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Syntheses and Spectral Properties of Longwave Absorbing and Fluorescing Substrates for the Direct and Continuous Kinetic Assay of Carboxylesterases, Phosphatases, and Sulfatases

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Syntheses, absorption and fluorescence properties for a series of new enzyme substrates are described, which are derived from 7-hydroxycoumarins possessing electron-withdrawing substituents in position 3. The new substrates are advantageous over existing ones in that they exhibit longwave absorption and fluorescence maxima as well as large *Stokes'* shifts. In addition, their pK_a values, which are usually between 6.0 and 7.0, allow the direct and continuous kinetic assay of hydrolases such as esterases, phosphatases, and sulfatases.

(Keywords: Enzyme substrates; Absorption spectra; Fluorescence spectra)

Synthesen und spektrale Eigenschaften langwellig absorbierender und fluoreszierender Substrate für die direkte und kontinuierliche kinetische Bestimmung von Carboxylesterasen, Phosphatasen und Sulfatasen

Die Synthesen und Absorptions- sowie Fluoreszenzspektren einer Reihe neuer Enzymsubstrate werden beschrieben, welche von 7-Hydroxycumarinen mit elektronenziehenden Substituenten in 3-Stellung abgeleitet sind. Die neuen Substrate bieten gegenüber bisherigen den Vorteil langwelliger Absorptions- und Fluoreszenzmaxima sowie großer *Stokes*-Verschiebungen. Die niedrigen pK_s-Werte, welche zwischen 6 und 7 liegen, erlauben weiters eine direkte und kontinuierliche kinetische Bestimmung von Hydrolasen vom Typ der Esterasen, Phosphatasen und Sulfatasen.

Introduction

Various esters of 7-hydroxy-4-methylcoumarin (4-methylumbelliferone, "*4-MU*") have been prepared in the last years and have been used as fluorogenic enzyme substrates[1,2]. Carboxylic acid esters, the phosphate and sulfate are commercially available and have found widespread

application, since they are virtually nonfluorescent by themselves, but are enzymatically cleaved to give *4-MU*. The latter, due to its pK_a of 7.8, undergoes partial dissociation at physiological *pH* to give the highly fluorescent *4-MU* anion. When fluorescence excitation is performed at 360 nm – a spectral region where almost exclusively the anion absorbs light – the increase in fluorescence with time is a measure for the enzymatic activity.

An inherent disadvantage of *4-MU* is its UV excitation. Most biological liquids such as human serum, intracellular liquids and urine display strong intrinsic fluorescence under 350 nm excitation, thus giving rise to strong background signals. A second disadvantage of *4-MU* is its relatively high pK_a, which causes only around 30% of *4-MU* to dissociate at *pH* 7.4. A phenolic compound of lower pK_a is therefore desired.

In continuation of our search for longwave fluorescent enzyme substrates[3] we perceived that both a longwave spectral shift and a decrease in the pK_a may be achieved by introducing electron withdrawing substituents in position 3 of the coumarin ring. Their bathochromic effect has already found application in the chemistry of optical brightheners and laser dyes[4]. The synthesis of esters of 3-substituted coumarins, which are useful enzyme substrates, along with an unusual effect of a 4-cyano group will be reported here.

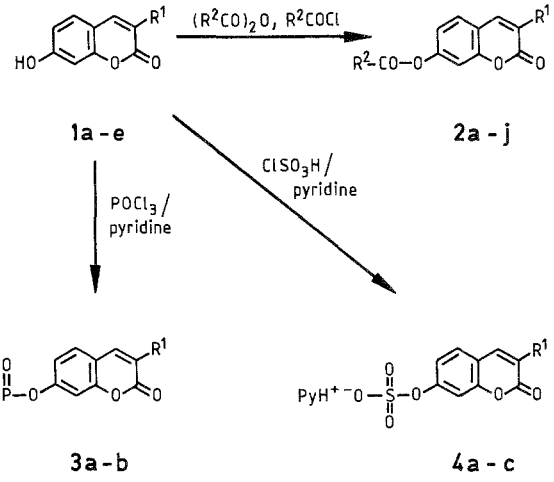
Results

We first performed a study on the spectral and acid-base properties of 7-hydroxycoumarins **1 a–e** bearing substituents in position 3. Their structures are given in Scheme 1. Table 1 shows that all excitation maxima and most emission maxima of coumarins **1 a–e** are at distinctly longer wavelengths than those of *4-MU*. Their pK_a values are also lowered by nearly one unit as compared with *4-MU* (7.8), expect for **1 e**, and their molar absorbances are higher. Therefore, esters of **1 a–e** seemed worth to be synthesized in order to obtain new substrates for the photometric and fluorimetric assay of clinically important enzymes.

Preparation of esters **2 a, b, d, j** was accomplished by reaction of the respective 7-hydroxycoumarin **1 a, b, d** with either acetic acid anhydride or butyric acid anhydride. The acetates and butyrate are obtained in high yield and purity. Caprylate **2 c** was obtained from **1 a** and caprylic acid anhydride, which was prepared *in situ* from caprylic acid and dicyclohexylcarbodiimide ("*DCC*"). Esters **2 e–i** were synthesized by reacting **1 c** with either the respective carboxylic acid anhydride or chloride in pyridine.

Phosphates **3 a, b** were prepared by analogy to the method applied to other 7-hydroxycoumarins[5,6], but were isolated as the more readily available pyridinium salts instead of the sodium salts, which can be

Scheme 1



1	R^1
a	2-benzoxazolyl
b	2-benzothiazolyl
c	2-(5-methyl-7-sulfonatobenzoxazolyl) pyridinium salt
d	COOH
e	phenyl

3	R^1
a	2-benzoxazolyl
b	phenyl

2	R^1	R^2-CO
a	2-benzoxazolyl	acetyl
b	2-benzoxazolyl	butyryl
c	2-benzoxazolyl	capryl
d	2-benzothiazolyl	acetyl
e	2-(5-methyl-7-sulfonato-benzoxazolyl) pyridinium salt	acetyl
f	2-(5-methyl-7-sulfonato-benzoxazolyl) pyridinium salt	butyryl
g	2-(5-methyl-7-sulfonato-benzoxazolyl) pyridinium salt	capryl
h	2-(5-methyl-7-sulfonato-benzoxazolyl) pyridinium salt	lauryl
i	2-(5-methyl-7-sulfonato-benzoxazolyl) pyridinium salt	oleyl
j	COOH	acetyl

4	R^1
a	2-benzoxazolyl
b	2-benzothiazolyl
c	phenyl

Table 1. *Longestwave absorption and fluorescence maxima (in nm) of 7-hydroxycoumarins* **1a–e** *and* **5a–c** *in acidic and alkaline solutions, as well as* pK_a *values. Data for 7-hydroxy-4-methylcoumarin (4-MU) are added for comparison*

Coumarin	*pH* Value of Aqueous Solution	Maximum of Absorption	Maximum of Fluorescence	ε ($M^{-1}\,cm^{-1}$)	pK_a Value[a] (23 °C)
4-MU	3	321	454[b]	14700	7.8[c]
	10	362	452	18900	
1a	3	377	469[b]	28200	6.84 ± 0.04
	10	427	471	44300	
1b	3	385	485[b]	31000	7.02 ± 0.02
	10	439	490	47000	
1c	3	378	468[b]	32000	6.80 ± 0.08
	10	431	470	44000	
1d	3	342	447[b]	15700	7.04 ± 0.02
	10	386	448	25100	
1e	3	338	465[b]	19700	7.80 ± 0.05
	10	383	472	26100	
5a	3	432	594[b]	23300	6.38 ± 0.03
	10	505	595	33100	
5b	3	418	576[b]	21400	6.00 ± 0.04
	10	497	577	32400	
5c	3	416	574[b]	22100	6.08 ± 0.04
	10	494	577	31600	

[a] pK_a values were determined by spectrophotometry in aqueous solutions containing 10% methanol.

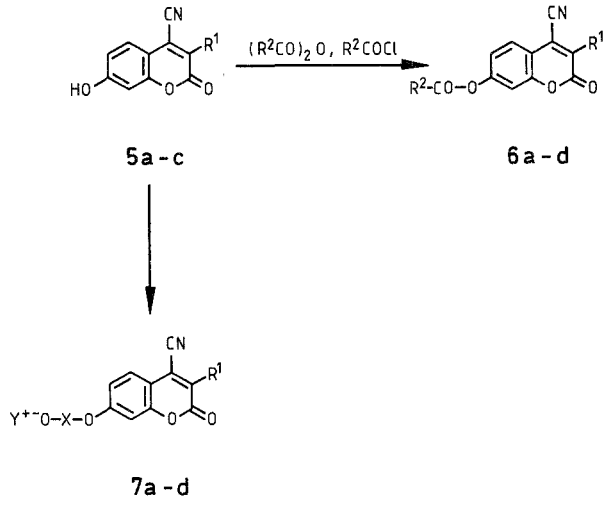
[b] Despite of photoexciting the neutral species, fluorescence is from the phenolate form due to photodissociation.

[c] From Ref.[8].

purified only with difficulty. Thus, **3a,b** were obtained from the appropriate 7-hydroxycoumarin with phosphorus oxytrichloride in pyridine at 0 °C. They were isolated as the semi-pyridinium salts from solutions of *pH* 6. When the pH value is lowered, the product has a different stoichiometry and contains more of the free acid. Careful *pH* control is therefore required. Although the yield of the crude product is usually high (60–85%), we encountered considerable losses during the purification procedure, which is necessary to get a product of sufficient purity for enzyme assays. Yields of the pure substrates did not exceed 45%.

Sulfates **4a–c** are obtained in around 70% yield by reacting 7-hydroxycoumarins with chlorosulfonic acid in pyridine, a method that was applied to the preparation of the sulfate of *4-MU*[7]. With respect to yield and purity of the product, the isolation via the pyridinium salts is

Scheme 2



5	R^1
a	2-benzothiazolyl
b	2-(5-chlorobenzoxazolyl)
c	2-(5-methyl-7-sulfonatobenzoxazolyl) potassium salt

7	R^1	X	Y^+
a	2-benzothiazolyl	$P(O)O^-Na^+$	Na^+
b	2-(5-chlorobenzoxazolyl)	P(O)OH	PyH^+
c	2-(5-chlorobenzoxazolyl)	SO_2	PyH^+
d	2-benzothiazolyl	SO_2	PyH^+

6	R^1	R^2-CO
a	2-benzothiazolyl	acetyl
b	2-benzothiazolyl	butyryl
c	2-benzothiazolyl	oleyl
d	2-(5-methyl-7-sulfonato-benzoxazolyl) pyridinium salt	lauryl

Table 2. *Longestwave absorption and fluorescence maxima of substrates* **2**, **3**, **4**, **6** *and* **7**

Substrate	Absorption Maximum (nm)	ε ($M^{-1}\,cm^{-1}$)	Fluorescence Maximum[b] (nm)	Solvent
2a	356	25700	451	dioxane
2b	356	25800	450	dioxane
2c	356	25600	453	dioxane
2d	365	29200	465	dioxane
2e	356	30200	434	methanol
2f	357	30300	434	methanol
2g	357	28700	435	methanol
2h	356	30100	435	methanol
2i	357	28200	436	methanol
2j	332	12100	398	dioxane
3a	367	28200	449	water
3b	332	18200	429	water
4a	356	29350	454	water
4b	366	28100	466	water
4c	327	17200	424	water
6a	401	26400	505	dioxane
6b	402	24900	506	dioxane
6c	402	26800	503	dioxane
6d	383	17600	515	acetone
7a	418	22900	534	water
7b	411	26300	522	water
7c	393	19300	516	water
7d	401	18650	498[a]	water

[a] Very weak.
[b] Under excitation at the absorption maximum.

again advantageous over the isolation of alkali metal salts. In fact, pyridinium salts **4a** and **4b** were obtained in excellent purity from the reaction mixture and required no further purification.

It has been noticed that introducing a cyano group into position 4 of 7-aminocoumarins[9] or umbelliferones[10] cause dramatic shifts in fluorescence maxima. As can be seen from Table 1, the fluorescence maxima of 4-cyano-7-hydroxycoumarins are at much longer wavelengths (577–595 nm) than those of the respective coumarins without a cyano group. Simultaneously, the pK_a values are lower by around 0.5 units. In addition, the *Stokes'* shifts are much larger (80–90 nm) than those of coumarins **1a–d** (39–62 nm). Only **1e** has a comparable shift (89 nm).

Consequently, we have also prepared esters of various 4-cyano-7-hydroxycoumarins (**5a–c**) with acetic, butyric, lauric and oleic acid (**6a–d**)

Table 3. *Yields and properties of enzyme substrates* **2**, **3**, **4**, **6** *and* **7**

Substrate	Formula (M.W.)	M.p. (°C)	Yield (%)	Recrystallization from
2a	$C_{18}H_{11}NO_5$ (321.29)	203–205	75	dioxane
2b	$C_{20}H_{15}NO_5$ (349.34)	193–195	77	dioxane
2c	$C_{24}H_{23}NO_5$ (405.45)	173–175	64	ethanol
2d	$C_{18}H_{11}NO_4S$ (337.34)	270–272	70	dioxane
2e	$C_{24}H_{18}N_2O_8S$ (494.49)	256[a]	71	ethanol
2f	$C_{26}H_{22}N_2O_8S$ (522.54)	210[a]	65	acetone/ethanol
2g	$C_{30}H_{30}N_2O_8S$ (578.65)	162[a]	58	acetone/ethanol
2h	$C_{34}H_{38}N_2O_8S$ (634.76)	135[a]	55	acetone
2i	$C_{40}H_{48}N_2O_8S$ (716.90)	102[a]	47	acetone
2j	$C_{12}H_8O_6$ (248.20)	208	72	acetic acid
3a	$C_{16}H_{10}NO_7P$[b] (398.79)	191–195[a]	37	ethanol (charcoal)
3b	$C_{15}H_{11}O_6P$[b] (357.78)	170	42	ethanol
4a	$C_{21}H_{14}N_2O_7S$ (438.42)	201–202	70	–
4b	$C_{21}H_{14}N_2O_6S_2$ (449.44)	203–205	60	–
4c	$C_{20}H_{15}NO_6S \cdot 2H_2O$ (433.44)	139	76	water
6a	$C_{19}H_{10}N_2O_4S$ (362.35)	256–258	77	dioxane
6b	$C_{21}H_{14}N_2O_4S$ (390.41)	244–246	80	dioxane
6c	$C_{35}H_{40}N_2O_4S$ (584.75)	163–165	65	dioxane
6d	$C_{35}H_{37}N_3O_8S$ (659.77)	89[a]	56	acetone[c]
7a	$C_{17}H_7N_2O_6PSNa_2 \cdot 8H_2O$ (588.41)	325 (dec.)	26	ethanol/water (4:1, *v/v*)
7b	$C_{17}H_8N_2O_7PCl$[d] (497.79)	195[a]	32[e]	ethanol/water (4:1, *v/v*)
7c	$C_{22}H_{12}N_3O_7SCl$ (497.88)	199–202	67	ethanol/water (1:4, *v/v*)
7d	$C_{22}H_{13}N_3O_6S_2$ (491.59)	222–232	65	ethanol/water (1:4, *v/v*)

[a] Sintering.
[b] Plus 1/2 pyridine.
[c] Cool to −15 °C.
[d] Plus 1 mol pyridine.
[e] Prepared as described for **7a**, but without neutralizing the aqueous solution with sodium hydroxide.

as well as with phosphoric and sulfuric acid (**7 a–d**) (see Scheme 2). Synthesis of phosphates and sulfates was rendered difficult due to the instability of 4-cyanocoumarins in alkaline solutions. Compound **7 a** – the first one prepared in this series – was isolated as the disodium salt. However, it was noted that addition of strong hydroxide solution to neutralize the phosphoric acid monoester resulted in considerable decomposition. Therefore, **7 b** was prepared as the monopyridinium salt, which can easily be isolated from weakly acidic solutions.

All compounds **6** and **7** possess a native fluorescence in the green (λ_{max} 500–534 nm) when excitation is near the absorption maximum (Table 2). The structures of all substrates **2–4**, **6** and **7** were confirmed by elemental analysis, IR-, ^{1}H-NMR and mass spectra. Their properties are compiled in Table 3.

Discussion

All enzyme substrates **2 a–j**, **3 a, b** and **4 a–c** have absorption maxima in the ultraviolet (Table 2) and exhibit weak blue fluorescence. Enzymatic hydrolysis (by esterases, phosphatases and sulfatases) converts the substrates into the highly fluorescent anions of the respective 7-hydroxycoumarins at physiological *pH*'s[11]. As shown in Table 1, their pK_a's are mostly lower than that of 7-hydroxycoumarin itself (7.8), and both the fluorescence excitation and emission maxima are in the visible, except for compounds **1 d** and **1 e**. Thus, when fluorescence excitation is performed at the wavelength of maximal anion absorption, only negligible fluorescence will be observed unless the substrates are cleaved by an enzyme.

The fairly high molar absorbance of the phenolates of coumarins **1 a–c** ($\varepsilon \sim 45\,000\, M^{-1}\,cm^{-1}$) is advantageous for both the photometric and fluorimetric enzyme assay. Carboxylic acid esters **2 e–i** derived from coumarin **1 c** bear a sulfonato group sufficiently distant from the reaction center. Consequently, they exhibit good water solubility and therefore can be used for the determination of carboxylic ester hydrolases such as lipases. Addition of an organic solvent to achieve water solubility is not required.

Although substrates **6** and **7** exhibit yellow absorption and a green fluorescence (Table 2), this does not interfere with the enzyme assay: When cleaved by an appropriate enzyme, the resulting 7-hydroxycoumarin will dissociate at near neutral *pH* to give the phenolate with its high molar absorbance and extremely longwave fluorescence, whose increase with time is followed. Spectral data of the phenolates, and thus the wavelengths at which photometric or fluorimetric enzyme determination should be performed, are given in Table 1.

Cyanocoumarins **5 a–c** are of particular usefulness in possessing pK_a values as low as around 6, which allows a continuous kinetic assay of

enzymes, even when the optimal *pH* of the reaction comes to lie below 7. It is therefore possible to perform direct and continuous kinetic assays of enzymes with maximal activity at *pH* values between 5 and 7, such as certain phosphatases or arylsulfatases.

The determination can be performed in a longwave spectral range, where no background fluorescence from biological material has to be expected. In addition, a large *Stokes'* shift (80–90 nm) can prevent interferences from *Raman* scatter and straylight. In conclusion, we think to have prepared and characterized a useful new class of enzyme substrates with properties superior to existing ones.

Experimental

All 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 A 60 A (^{1}H-NMR); Varian Mat 111 (mass spectra). Elemental analyses were performed on a Carlo-Erba 1106 C,H,N-analyzer, and *pH* determinations with a glass electrode (Metrohm, Switzerland) that was calibrated against *pH* 7 and 4 standard buffers.

7-Acetoxy-3-(2-benzoxazolyl)coumarin (**2a**)

To 5 ml dimethylformamide 0.7 g (2.5 mmol) 3-(2-benzoxazolyl)-7-hydroxycoumarin and 4 ml (4.3 g, 40 mmol) acetic acid anhydride were added. The mixture was heated to 60 °C for 3 h under exclusion of moisture. The yellow crystals precipitating from the clear solution on cooling were collected and recrystallized from dioxane. The properties are given in Table 3.

$C_{18}H_{11}NO_5$ (321.29). Calc C 67.29 H 3.45 N 4.36.
Found C 67.49 H 3.36 N 4.34.

Compounds **2b–d, j** were prepared similarly from the corresponding 7-hydroxycoumarin and either acetic acid anhydride, butyric acid anhydride or caprylic acid anhydride. The latter was prepared in situ from two moles of caprylic acid and one mole of dicyclohexylcarbodiimide and was directly used after filtering off the precipitate (dicyclohexylurea). Table 3 gives the properties of the resulting compounds.

7-Capryloxy-3-[2-(5-methyl-7-sulfonatobenzoxazolyl)]coumarin pyridinium salt (**2g**)

A mixture of 0.45 g (1 mmol) 7-hydroxy-3-[2-(5-methyl-7-sulfonatobenzoxazolyl)]coumarin pyridinium salt, 0.5 g (3 mmol) caprylic acid chloride – or 0.8 g (3 mmol) caprylic acid anhydride – and 1 ml dry pyridine was heated under reflux for 5 min. After addition of 5 ml acetone the precipitate was collected and recrystallized from a mixture of acetone and ethanol. Data in Table 3.

$C_{30}H_{30}N_2O_8S$ (578.65). Calc C 62.27 H 5.23 N 4.84.
Found C 62.54 H 5.35 N 4.55.

Compounds **2e, f, h, i** were prepared in a similar way, starting from 7-hydroxy-3-[2-(5-methyl-7-sulfonatobenzoxazolyl)]coumarin pyridinium salt and either the respective acid anhydride or acid chloride. Their properties are given in Table 3.

7-(3-Phenylcoumarinyl)phosphate semi-pyridinium salt (**3b**)

To an ice-cooled mixture of 10 ml dry pyridine and 0.46 ml (0.77 g, 5 mmol) phosphorus oxytrichloride 1.19 g (5 mmol) 7-hydroxy-3-phenylcoumarin were added within 30 min. Stirring at 0 °C was continued for 1 h. After addition of 200 ml cold water and filtration the solution was evaporated to dryness under reduced pressure. The residue was recrystallized from ethanol and dried over phosphorus pentoxide. Yield and properties in Table 3.

$C_{15}H_{11}O_6P \cdot 1/2$ pyridine (357.78). Calc C 58.75 H 3.80 N 1.96.
Found C 58.61 H 3.78 N 1.94.

3a was obtained by the same procedure, starting from 3-(2-benzoxazolyl)-7-hydroxycoumarin and $POCl_3$ in a molar ratio of 1 : 1. Data in Table 3.

3-(2-Benzoxazolyl)-7-coumarinylsulfate pyridinium salt (**4a**)

To 5 ml dry pyridine 0.82 ml (1.47 g, 12.5 mmol) chlorosulfonic acid was slowly dropped under cooling and stirring. After addition of 0.7 g (2.5 mmol) 3-(2-benzoxazolyl)-7-hydroxycoumarin the reaction mixture was heated to 60 °C for 24 h under exclusion of moisture. After adding 20 ml water the suspension was filtered at 90 °C. The yellow crystals separating from the filtrate upon cooling were collected and dried over phosphorus pentoxide. Properties in Table 3.

$C_{21}H_{14}N_2O_7S$ (438.42). Calc C 57.53 H 3.22 N 6.39 S 7.31.
Found C 57.58 H 3.17 N 6.54 S 7.07.

Compounds **4b, c** were prepared by analogy to this procedure. Their properties are given in Table 3.

3-(2-Benzothiazolyl)-7-butyryloxy-4-cyanocoumarin (**6b**)

In 50 ml dimethylformamide, 3.0 g (9.4 mmol) 3-(2-benzothiazolyl)-4-cyano-7-hydroxycoumarin and 10 ml (9.7 g, 60 mmol) butyric acid anhydride were heated to 70 °C for 8 h under exclusion of moisture. The resulting precipitate was collected and recrystallized from dioxane. Yield and properties in Table 3.

$C_{21}H_{14}N_2O_4S$ (390.41). Calc C 64.60 H 3.61 N 7.18 S 8.21.
Found C 64.79 H 3.65 N 7.11 S 7.98.

Compounds **6a, c** were obtained by analogy. Compound **6d** was prepared by the same method as given for **2g**. Properties in Table 3.

3-(2-Benzothiazolyl)-4-cyano-7-coumarinylphosphate disodium salt (**7a**)

To a cooled mixture (0 °C) of 10 ml dry pyridine and 0.23 ml (0.38 g, 2.5 mmol) phosphorus oxytrichloride 0.8 g (2.5 mmol) 3-(2-benzothiazolyl)-4-cyano-7-hydroxycoumarin were added within 30 min under stirring, which was continued after addition of reagent for 1 h. Then, 200 ml cold water were added and the solution was adjusted to *pH* 7 with 10 *N* sodium hydroxide. After filtration, it was rapidly evaporated under reduced pressure at a temperature below 50 °C. The residue was recrystallized from ethanol/water (4 : 1, *v/v*) and the resulting orange crystals were dried in air. Data in Table 3.

$C_{17}H_7N_2O_6PSNa_2 \cdot 8H_2O$ (588.41). Calc C 34.70 H 3.94 N 4.76 S 5.45.
Found C 34.94 H 3.67 N 4.76 S 5.96.

3-(2-Benzothiazolyl)-4-cyano-7-coumarinylsulfate pyridinium salt (**7 d**)

To 5 ml dry pyridine 0.32 ml (0.57 g, 5 mmol) chlorosulfonic acid was slowly dropped under cooling and stirring. After addition of 0.80 g (2.5 mmol) 3-(2-benzothiazolyl)-4-cyano-7-hydroxycoumarin the reaction mixture was heated to 60 °C for 2 h under exclusion of moisture. 40 ml cold water were added after chilling the solution. The resulting precipitate was rapidly collected and recrystallized from water/ethanol (4 : 1, *v/v*). The orange crystals were dried over phosphorus pentoxide.

$C_{22}H_{13}N_3O_6S_2$ (491.59). Calc C 53.75 H 2.67 N 8.55 S 13.04.
Found C 53.71 H 2.71 N 8.59 S 12.44.

7 c was prepared by the same procedure, starting from **5 b**. Data in Table 3.

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References

[1] *Guilbault G. G.*, Anal. Chem. **42**, 334 R (1970).
[2] *Kanaoka Y.*, Angew. Chem. **89**, 142 (1977); Angew. Chem. Int. Edit. Engl. **16**, 137 (1977).
[3] *Wolfbeis O. S., Koller E.*, Anal. Biochem. **129**, 365 (1983).
[4] *Dorlars A., Schellhammer C. W., Schroeder J.*, Angew. Chem. **87**, 693 (1975); Angew. Chem. Int. Edit. Engl. **14**, 665 (1975).
[5] *Fernley H. N., Walker P. G.*, Biochem. J. **97**, 95 (1965).
[6] *Guilbault G. G., Sadar S. H., Glazer R., Haynes J.*, Anal. Lett. **1**, 333 (1968).
[7] *Sherman W. R., Stanfield E. F.*, Biochem. J. **102**, 905 (1967).
[8] *Yakatan G. J., Juneau R. J., Schulman S. G.*, Anal. Chem. **44**, 1044 (1972).
[9] *Moeckli P.*, Dyes and Pigments **1**, 3 (1980), and references cited therein.
[10] *Wolfbeis O. S., Koller E., Hochmuth P.*, Bull. Chem. Soc. Jpn., in press.
[11] To be published separately. See, for instance: *Koller E., Wolfbeis O. S.*, Anal. Biochem., in press.