Monatshefte für Chemie 118, 1403—1411 (1987)

Monatshefte für Chemie
Chemical Monthly

The Fluorescence Properties of Luteolines

Otto S. Wolfbeis[a,*], **Monowara Begum**[a], and **Hans Geiger**[b]

[a] Institut für Organische Chemie, Karl-Franzens-Universität, A-8010 Graz, Austria

[b] Institut für Chemie, Universität Hohenheim, D-7000 Stuttgart 70, Federal Republic of Germany

(Received 13 October 1986. Accepted 20 November 1986)

The fluorescence properties of luteoline and its possible methyl ethers (a representative class of naturally occurring flavonoids) were investigated, together with the effect of diagnostic shift reagents such as sulfuric acid, aluminium trichloride, and borax. The results demonstrate that fluorimetry is a suitable tool for the identification and structure elucidation of minute samples of flavones, in particular if combined with absorption spectrometry. The pK_a values of all trimethylethers were determined for the ground and first excited singlet states and related to the effects of basic reagents.

(Keywords: Flavones; Fluorescence; Dissociation constants; Structure elucidation)

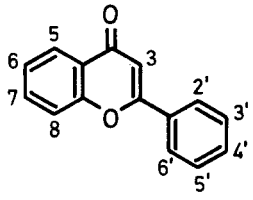
Die Fluoreszenzeigenschaften der Luteoline

Es wurden die Fluoreszenzeigenschaften der Luteoline (einer repräsentativen Klasse von natürlich vorkommenden Flavonoiden) sowie der Effekt diagnostischer Verschiebungsreagentien wie z. B. Schwefelsäure, Aluminiumtrichlorid und Borax untersucht. Die Ergebnisse zeigen, daß die Fluorimetrie eine geeignete Methode zur Identifikation und Strukturaufklärung auch geringster Probenmengen sein kann, besonders in Kombination mit Absorptionsmessungen. Die pK_s-Werte aller möglichen Trimethylether wurden für Grund- und ersten angeregten Zustand bestimmt und zu den Effekten der basischen Reagentien in Bezug gebracht.

Introduction

Luteoline (5,7,3′,4′-tetrahydroxyflavone) and its methyl ethers are a group of naturally occurring flavones. They represent a major class of organic natural products [1, 2]. The chemical structure of flavone (2-phenyl-4*H*-benzopyran-4-one) is shown below, along with the numbering system. We have chosen the luteolines as suitable model compounds for performing a study on the spectral properties of natural flavones, because both luteoline and all its possible methyl ethers were available.

Reports on the fluorescence of natural flavones are scarce. Apart from early observations [3] and previous systematic work on the fluorescence of flavonoides on TLC [4, 5] and in solution [7] there are only a few published studies available, dealing mainly with model compounds [8].



The aim of the present work was a systematic study on the spectral properties of this representative group of flavonoids with respect to the substitution pattern and the effect of diagnostic reagents on the emission spectra.

Since basic reagents such as sodium acetate are useful to discern between differently substituted hydroxyflavones by their different dissociation constants, the pK_a values of a representative number of isomeric flavones were determined for the first time. The results were expected to give more insight into the relation between fluorescence properties of flavonoids, and why diagnostic "shift reagents" make possible a fairly unambiguous structure elucidation by spectrometry.

Experimental

Samples. Luteoline and its methyl ethers were isolated from natural sources or synthesized by established methods. They were purified by thin layer chromatography and/or recrystallization until they did not contain any TLC-visible impurities. Their identity was proven by correct melting points and absorption spectra in accordance with literature data.

Reagents. All solvents were purified (or from the same source) as described previously [7]. In essence, these are (a) 50% sulfuric acid, (b) 5% sodium acetate in water, (c) 1% sodium hydrogencarbonate in water, (d) 1% anhydrous aluminium trichloride in methanol, and (e) 1% borax in water. Methanol stock solutions (ca. 10^{-4} *M*) were prepared and used for further experiments. For fluorescence studies, 3 ml of the stock solution were transferred into a quartz cell and the emission spectrum recorded. Then, 1 ml of the "shift" reagent was added and the spectrum run again. An exception was made when water (3 ml) was added to the methanol stock (1 ml only).

pK_a Determinations. pK_a values were determined by the spectrophotometric method at several analytical wavelengths in the 350–390 nm range. The ionic strength of the solutions was kept below 0.05. 10 ml of the methanol stock solutions were diluted to 100 ml with water and 1 ml of a buffer having buffer capacity near the pK_a of the compound (phosphate, *TRIS*, or bicarbonate/NaOH) was added.

The *pH* of the solution was adjusted to about 4 units below the expected pK_a value by addition of 0.1 *N* hydrochloric acid. Titration was performed with 0.01 *N* or 0.001 sodium hydroxide solutions. After adding the base the solution was carefully stirred. A sample was then transferred into the quartz cell and its optical density measured with the photometer. pK_a's were determined from absorbance data by the known method [9].

Instrumentation.—Absorption spectra were run on a Perkin-Elmer Lambda 5 instrument and fluorescence spectra on an Amino SPF 500 fluorimeter having a 250-Watt xenon light source. Fluorescence was excited at the respective absorption maximum of the luteoline. Bandpasses were between 5 and 10 nm in both excitation and emission. Larger bandpasses are possible for compounds displaying large *Stokes'* shifts between excitation and emission. Optical density data for pK_a determinations were obtained on a Zeiss PMQ II photometer at fixed wavelengths. *pH*'s were measured with a Metrohm model 632 *pH* meter that was calibrated at *pH* 7.00 and 10.00.

Results

Spectral Data

As known from other 5-hydroxyflavones, luteoline itself is almost nonfluorescent in methanol and in water, showing an emission of minute intensity at λ_{max} 433 nm (in methanol). Neither sulfuric acid (10% or 50% in water), or basic reagents (bicarbonate or carbonate) cause an increase in intensity. If, however, the 5-hydroxy group is replaced by a methoxy group, fluorescence becomes considerably more intense. Generally it is observed that fluorescence increases with the number of hydroxy groups being methylated.

Table 1 summarizes the spectral maxima of the luteolines investigated with relative intensities given in brackets. The scale (1–6) is based on qualitative quantum yield estimations. Fluorescence can visually be detected in the 6-3 intensity range, whereas 2 and 1 indicate that fluorescence detection is possible with an instrument only. In terms of quantum yields, 6 and 5 represent values from, roughly, 0.1 to 1.0, and 2 and 1 quantum yields of below 0.002.

The spectral maxima of the luteolines after addition of various reagents are also given in Table 1. As with luteoline itself, all derivatives possessing a free 5-hydroxy group are virtually nonfluorescent (entries 1, 3, 4, 5, 9, 10, 11, 15) as indicated by the low figures gives in brackets. With the 5-hydroxy group methylated, the emission intensity is increased, reaching a maximum with the fully methylated derivative (entry 16).

Addition of water shifts the maxima to longer wavelengths. Sometimes an additional weak emission band is found at above 500 nm. The green band is observed only when a 7-hydroxy group is present. It becomes even more intense after addition of sodium acetate (see data in Table 1).

10% Sulfuric acid does not cause significant spectral changes useful

Table 1. *Fluorescence maxima (in nm) of luteoline and its methyl ethers in methanol and the effect of diagnostic reagents. Relative intensities, ranging from 1 (very low) to 6 (very high) are given in brackets*

Entry	hydroxy group in	Fluorescence maximum in						
		*Me*OH	water	+50% H_2SO_4	+NaO*Ac*	+$NaHCO_3$	+$AlCl_3$	+borax
1	5,7,3′,4′	433 (1)	440 (1)	438 (2)	440, 511 (1)	440, 511 (2)	540 (4)	436, 520 (1)
2	7,3′,4′	453 (4)	451 (4)	500 (4)	508 (4)	506 (4)	531 (4)	500 (5)
3	5,3′,4′	430 (1)	440 (1)	460 (1)	430	515 (2)	545 (3)	430, 500 (1)
4	5,7,4′	445 (1)	460 (2)	510 (2)	440, 507 (2)	508 (2)	520 (3)	512 (4)
5	5,7,3′	430 (2)	430 (2)	–	430, 512 (2)	512 (3)	523 (3)	433, 510 (2)
6	3′,4′	457 (4)	460 (3)	470, 520 (3)	460 (4)	510 (4)	525 (4)	530 (5)
7	7,4′	434 (5)	450 (4)	504 (4)	438, 500 (4)	500 (2)	440 (5)	503 (5)
8	7,3′	435 (5)	438 (4)	480 (4)	514 (4)	513 (4)	438 (4)	518 (4)
9	5,4′	436 (2)	420 (2)	512 (2)	420, 520 (2)	519 (2)	520 (3)	526 (2)
10	5,7	430 (1)	440 (1)	500 (2)	430, 510 (2)	513 (2)	523 (3)	430 (2)
11	5,3′	418 (1)	414 (1)	456 (3)	420 (1)	439 (2)	523 (3)	420, 505 (2)
12	4′	440 (4)	450 (5)	510 (5)	445 (4)	458, 520 (4)	445 (4)	454 (4)
13	3′	440 (5)	440 (4)	483 (4)	430 (4)	430 (2)	452 (4)	436 (4)
14	7	427 (5)	444 (4)	507 (4)	432, 520 (4)	517 (3)	432 (4)	518 (3)
15	5	435 (2)	450 (2)	510 (3)	440 (2)	440 (2)	444 (4)	440 (4)
16	–	432 (6)	445 (5)	515 (5)	445 (5)	445 (5)	435 (5)	440 (5)

for structure elucidation. The respective data have therefore not been included into Table 1. 50% sulfuric acid, in contrast, is strong enough to protonate, at least partially, all luteolines. As a result, the maxima suffer a shift of the fluorescence maximum to longer wavelengths, concomitant with an increase in intensity. Luteoline and its 7-methyl ether, however, are notable exceptions. Generally, the effect is least expressed with 5-hydroxyflavones. Luteolines having a 3′-hydroxy group suffer a considerable smaller spectral shift after addition of 50% acid (by 5–47 nm only) than those having a 3′-methoxy group (65–83 nm).

Sodium acetate (Table 1) produces the anion fluorescence of all luteolines possessing either a 7-hydroxy or 4′-hydroxy group, resulting in a shift of the emission maximum to beyond 500 nm. With sodium bicarbonate, all luteolines having a free 4′- or 7-hydroxy group fluoresce from the anion species, while those having free 3′- and/or 5-hydroxy groups (entries 11, 13, 15, 16) remain unaffected. The reason therefore is the relatively higher pK_a value for the latter derivatives.

The effect of sodium carbonate was also investigated but was found to give no additional information to bicarbonate. 0.1 *N* sodium hydroxide is known to cause a longwave spectral shift in the absorption spectra of all flavonoids possessing a free hydroxy group, but fluorescence is frequently quenched. The reagent is therefore of little diagnostic value in fluorimetry.

Among the complexing shift reagents, aluminium trichloride and borax are most useful. The former is a proper reagent for detection of 5-hydroxy groups in bringing about a yellow-green fluorescence of almost non-fluorescent 5-hydroxyflavones. A spectral shift indicates that the 3′,4′-dihydroxy group (entires 2 and 6) also becomes complexed by this reagent. $AlCl_3$ remains without effect upon 5-methoxy derivatives having a 3′- or 4′-methoxy group (entries 7, 8, 12–14, 16).

The effect of borax produces a strong green fluorescence with compounds possessing a 3′,4′-dihydroxy structure (entries 2, 6), except of course for the 5-hydroxy derivatives (entries 1 and 3). Possibly, other *ortho*-dihydroxy structures may also be recognized. *Neu*'s reagent which was found to be of high diagnostic value in previous work [5, 6] was not investigated because of the strong blue background fluorescence of its methanol solutions.

pK_a Determinations

It was obvious from the above results that there must be considerable differences in the pK_a values of the various hydroxy groups of luteolines. However, a determination of all pK_a's of luteoline itself deemed difficult in view of probably strongly overlapping values. Therefore, the four

Table 2. *Absorption maxima of luteoline trimethyl ethers in aqueous pH 5.0 and 0.1 N sodium hydroxide solutions, and photometrically determined pK_a values at 23 °C*

position of hydroxy group	position of methoxy groups	absorption maximum (nm) in *pH* 5.0	absorption maximum (nm) in 0.1 *N* NaOH	pK_a (S_0)	pK_a (S_1)	ΔpK_a
7	5,3′,4′	340	360	7.32 ± 0.05	−2.98[a]	−10.3
4′	5,7,3′	329	378	8.46 ± 0.07	1.18[a]	−7.3
3′	5,7,4′	338	363	9.26 ± 0.08	4.98[b]	−4.3
5	7,3′,4′	346	366	10.77 ± 0.12	7.45[b]	−3.3

[a] Calculated from the O−O-transitions obtained by averaging the wavenumbers of absorption and fluorescence maxima [11]
[b] Calculated from the absorption maxima, since no fluorescence of the anion was detectable

isomeric trimethyl ethers were chosen as candidates, each of them having one well defined dissociable group.

The pK_a values of the isomeric trimethylethers are compiled in Table 2. The data are surprising since the pK_a differences are much larger than may be expected. Thus, the 5,3′,4′-trimethylether of luteoline is about 2 500 times stronger an acid than the 7,3′,4′-isomer.

It is known that phenols, in their excited states, are stronger acids than in their respective ground state [10]. Excited state pK_a values [referred to as $pK_a(S_1)$ values] can be calculated [10] from absorption and fluorescence data according to the following equation:

$$pK_a(S_1) = pK_a(S_0) - 0.625\,(\nu_{HB} - \nu_B) \qquad (1)$$

Here, $pK_a(S_0)$ is the value for the ground state pK_a, ν_{HB} the O−O-transition for the conjugate acid (i.e., the phenol), and ν_B the O−O-transition for the conjugate base, i.e., the phenolate. The average of the wavenumbers (in cm^{-1}) of the absorption and fluorescence maxima were taken [11] as O−O-transitions to calculate the results, except for entries 3 and 4, where the absorption maxima had to be taken because of the lack of anion fluorescence.

The data, given in Table 2, show that indeed all isomeric luteoline trimethyl ethers become stronger acids in the S_1 state. The differences between ground state and excited state $pK_a(pK_a)$ vary considerably and are most pronounced for the 7-hydroxy isomer.

Discussion

The absence of appreciable fluorescence of luteolines possessing a 5-hydroxy group, together with the yellow fluorescence produced with $AlCl_3$ reagent is typical for 5-hydroxyflavones. 3-Hydroxyflavones, in contrast, display a weak greenish-yellow fluorescence which after addition of $AlCl_3$, is dramatically intensified and slightly shifted to shorter wavelengths. A 3,5-dihydroxyflavone would exhibit a yellow fluorescence as well which, however, is much less intense than that of a flavonol without a 5-hydroxy group [7]. Morin, the well known reagent for aluminium, may be cited as a typical example.

A differentiation between 5-hydroxy and 5-methoxy derivatives may be made by the *Kuhn-Löw* test [12]. In acetic anhydride solution only the 5-methyl ethers are fluorescent, but addition of B_2O_3 makes 5-hydroxy derivates fluorescent as well.

The appearance of weak anion fluorescence of some 7-hydroxy derivatives after the addition of water (data not given in the Table) is interpreted in terms of (a) the low pK_a of the 7-hydroxy group, and (b) adiabatic photodissociation in the first excited singlet state. It has been

shown for 7-hydroxyflavone [13] that photodissociation is the major deactivation path in neutral and weakly acidic solution. The calculation of the excited state pK_a value of luteoline-5,3′,4′-trimethyl ether (Table 2) predicts indeed an excited state pK_a as low as -2.98 and, therefore, photodissociation to occur.

Sodium acetate transforms all 7-hydroxy derivates into the monoanions with their fluorescence at, or beyond, 500 nm. One 4′-hydroxy derivatives (entry 9) is also affected by this reagent. The pK_a of the 4′-hydroxy group is that closest to the one of the 7-hydroxy group. The 4′-hydroxy group is therefore likely to be deprotonated by a slightly basic reagent such as sodium acetate, but in particular by bicarbonate (entries 3, 6, 9, 12). The 3′-hydroxy group may suffer dissociation following addition of bicarbonate solution, as indicated by UV spectrometry, but the corresponding anions seem to be nonfluorescent.

Borax is similar to sodium acetate as a slightly basic reagent. However, in the presence of a 3′,4′-dihydroxy group the spectral maxima are different from those observed with added acetate or bicarbonate, thus indicating a different type of interaction. Typical examples are entries 2 and 6. Complexation of a 3′,4′-dihydroxy group by borax also results in an strong increase in fluorescence intensity.

In summary, fluorescence spectroscopy seems to be a useful tool to identify positional isomers of minute amounts of flavonoids. Absorption spectroscopy, however, should be applied in every case before, or in combination with, fluorimetry. In practically all cases the maxima of absorption and fluorescence give a unique combination. Added shift reagents are useful in that they allow a differentiation to be made between various hydroxy group by virtue of their different pK_a values. Finally, complexing reagents allow the identifications of 5-hydroxy and 3′,4′-dihydroxy groups.

References

[1] *Harborne JB, Mabry TJ, Mabry H* (eds) (1975) The flavonoids. Chapman & Hall, London
[2] *Mabry TJ, Markham KR, Thomas MB* (1979) The systematic identification of flavonoids. Springer, Berlin Heidelberg New York
[3] *Harborne JB* (1959) J Chromatogr 2: 581
[4] *Spiegl P* (1969) J Chromatogr 39: 93
[5] *Homberg H, Geiger H* (1980) Phytochemistry 19: 2443
[6] *Geiger H, Homberg H* (1983) Z Naturforsch 38B: 253
[7] *Wolfbeis OS, Begum M, Geiger H* (1984) Z Naturforsch 39B: 231
[8] *Wolfbeis OS* (1985) The fluorescence of organic natural products. In: *Schulman GS* (ed) Molecular luminescence spectrometry: methods and applications – part 1. Wiley, New York, p 168–370
[9] *Eistert B, Reiss W* (1954) Ber Dtsch Chem Ges 87: 108

[10] For a review see: *Grabowski ZR, Grabowska A* (1976) Z Phys Chem 101: 197
[11] *Bartok W, Lucchesi P, Snider N* (1962) J Am Chem Soc 84: 1842
[12] *Kuhn R, Löw I* (1964) Ber Dtsch Chem Ges 77: 211
[13] *Schipfer R, Wolfbeis OS, Knierzinger A* (1981) J Chem Soc, Perkin Trans II, 1443