150. Synthesis and Properties of a New Dihydroflavin: Reduction of a Flavinium Salt by Borocyanohydride[1])

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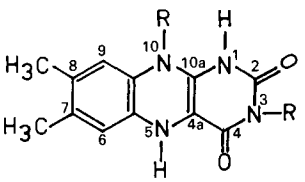
(2.V.90)

Reduction of **6** by borocyanohydride yielded the new dihydroflavin **7**. The intermediate product **8** could only be observed in solution by ^{1}H-NMR. The chemical and physical properties of **7** are reported. UV/VIS, ^{1}H- and ^{13}C-NMR, and luminescence techniques were used.

1. Introduction. – The two-electron-reduced flavin[4]) plays an important role in biological reactions catalyzed by flavoproteins. The most common and most important reduced form is 1,5-dihydroflavin (**1**). Catalytic or chemical reduction of oxidized flavin under mild conditions yields always **1** which is extremely air-sensitive. The reduction is thermodynamically fully reversible. In contrast, the re-oxidation of the more recently discovered isomeric dihydroflavins requires irradiation in the presence of O_2. The following isomeric



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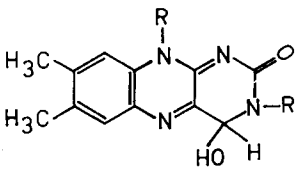

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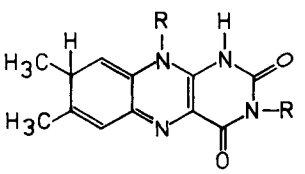

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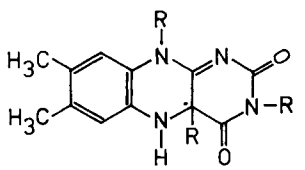

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[1]) Preliminary results were reported at the 'Fifth International Symposium on Flavins and Flavoproteins', San Francisco, March 31 – April 3, 1975.

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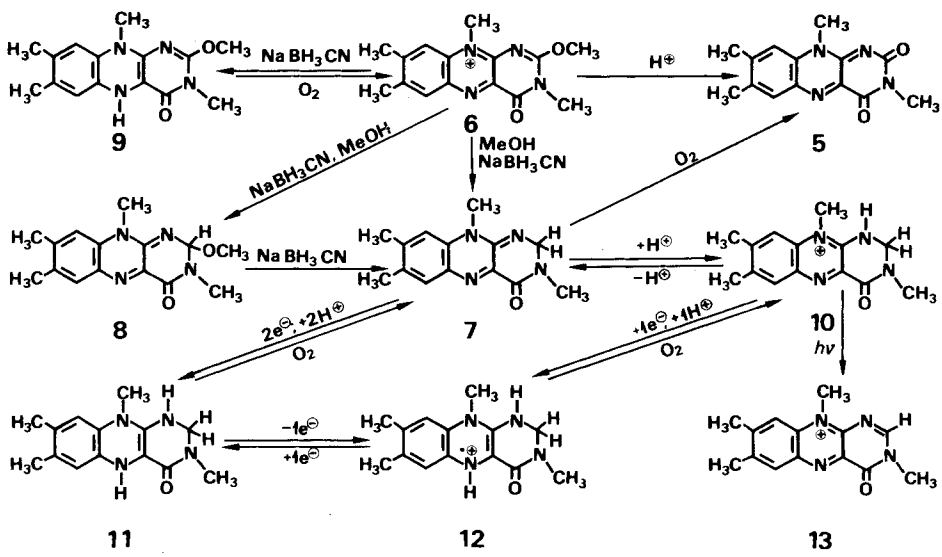
[4]) Flavin = 10-substituted 7,8-dimethylisoalloxazine = 10-substituted 7,8-dimethyl benzo[*g*]pteridine-2,4-(3*H*,10*H*)-dione.

dihydroflavins are known: 1,8-, 4a,5-, and 4,4-*O*-dihydroflavin [1–3], *i.e.* **2**, **3**, and **4**, respectively. These dihydroflavins are obtained from the oxidized molecule by photochemical reaction, the 4a,5-dihydroflavin can also be obtained by alkylation of 1,5-dihydroflavin in the dark [4].

In context with our interest in nucleophilic dark reactions of oxidized flavins as model reactions for biological systems [5–7], we observed that flavinium salts react with borocyanohydride to yield a new type of dihydroflavin. In a preliminary report [8], we have proposed a structure which was based solely on ^{1}H-NMR spectra of the reaction mixture. Recently, it has become evident that the structural assignment was ambiguous. We have now succeeded to isolate the reduction product in crystalline state, making it possible to elucidate its structure. In this paper, we describe the synthesis and the physical and chemical properties of the new dihydroflavin.

2. Results and Discussion. – Neutral oxidized flavin **5** (*Scheme*) reacts very slowly with borocyanohydride, yielding 1,5-dihydroflavin under anaerobic conditions. In the presence of light, 4,4-*O*-dihydroflavin is produced under aerobic conditions [3]. The structurally related alloxazines give also a 4,4-*O*-dihydro analogue by borohydride reduction, but the reaction proceeds smoothly in the absence of light [9].

Scheme



Borohydride reacts also very slowly with **6** to form **7** (*Scheme*). For this reason, no kinetic studies were undertaken. The preparative conversion of **6** into **7** leads to the formation of a considerable amount of **5** owing to the relatively easy hydrolysis of **6** [10] [11]. However, the higher basicity of **7** as compared to that of **5** makes it possible to separate the by-product quantitatively from the desired product (see *Exper. Part*). In addition, it should be noted that the neutral form of **7** decomposes slowly in the crystalline state and should, therefore, be transformed into the flavinium salt **10** which can be stored without noticeable deterioration for several months, as judged by TLC.

The reduction of **6** by borocyanohydride is expected to yield **8** (*Scheme*). The mass spectrum (M^+ 256) and the elemental analyses, however, indicate that the final product possesses structure **7** which is in full agreement with NMR data (see below). Nevertheless, **8** must be an intermediate in the synthesis of **7**, although attempts to isolate it failed. Evidence for the formation of **8** comes from ^{1}H-NMR data as shown in *Fig. 1* (see also *Table 1*). The structure of **7** was further ascertained by spectroscopy, using selectively

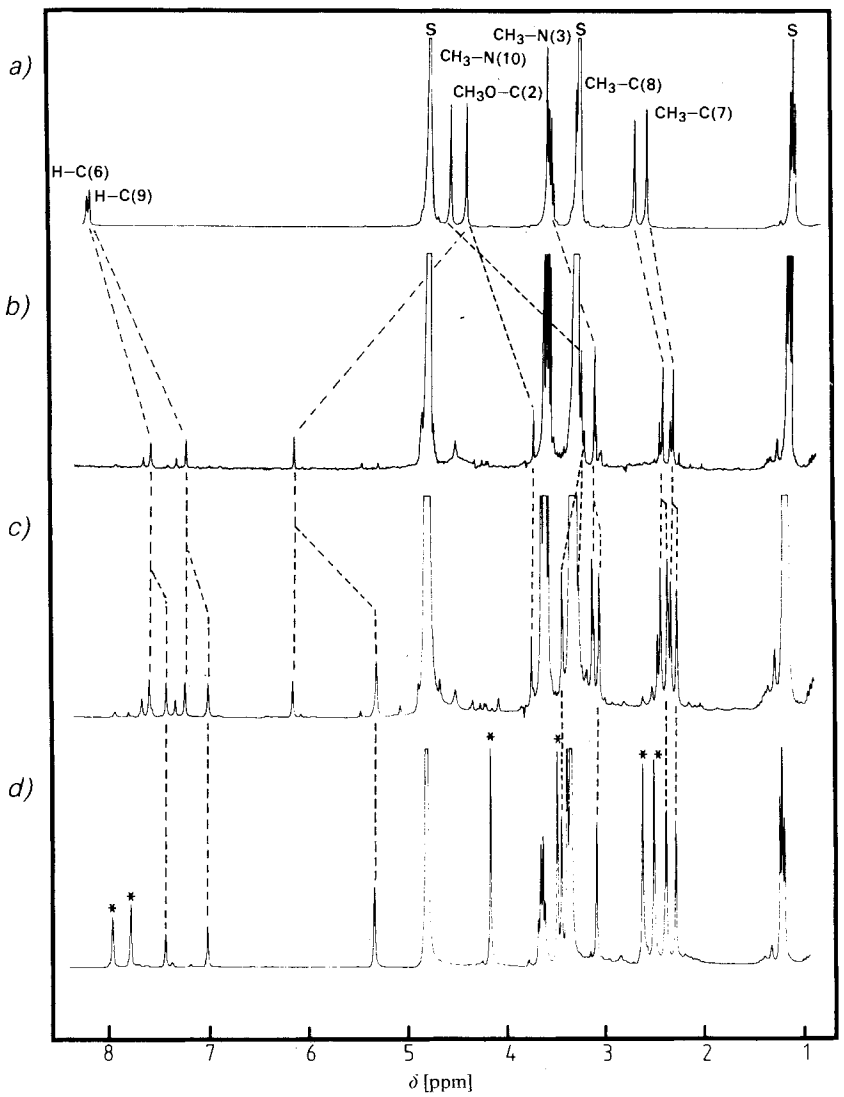


Fig. 1. *^{1}H-NMR spectra of* **6** *in CD_3OD* a) *in the absence of $NaBH_3CN$,* b) *immediately after addition of $NaBH_3CN$, and* c) *and* d) *20 min and 4 h, respectively, after addition of $NaBH_3CN$.* The resonances labelled with an asterisk are due to **5**, S denotes signals from the solvents CD_3OD and EtOH, the latter was associated with **6** freshly crystallized from EtOH.

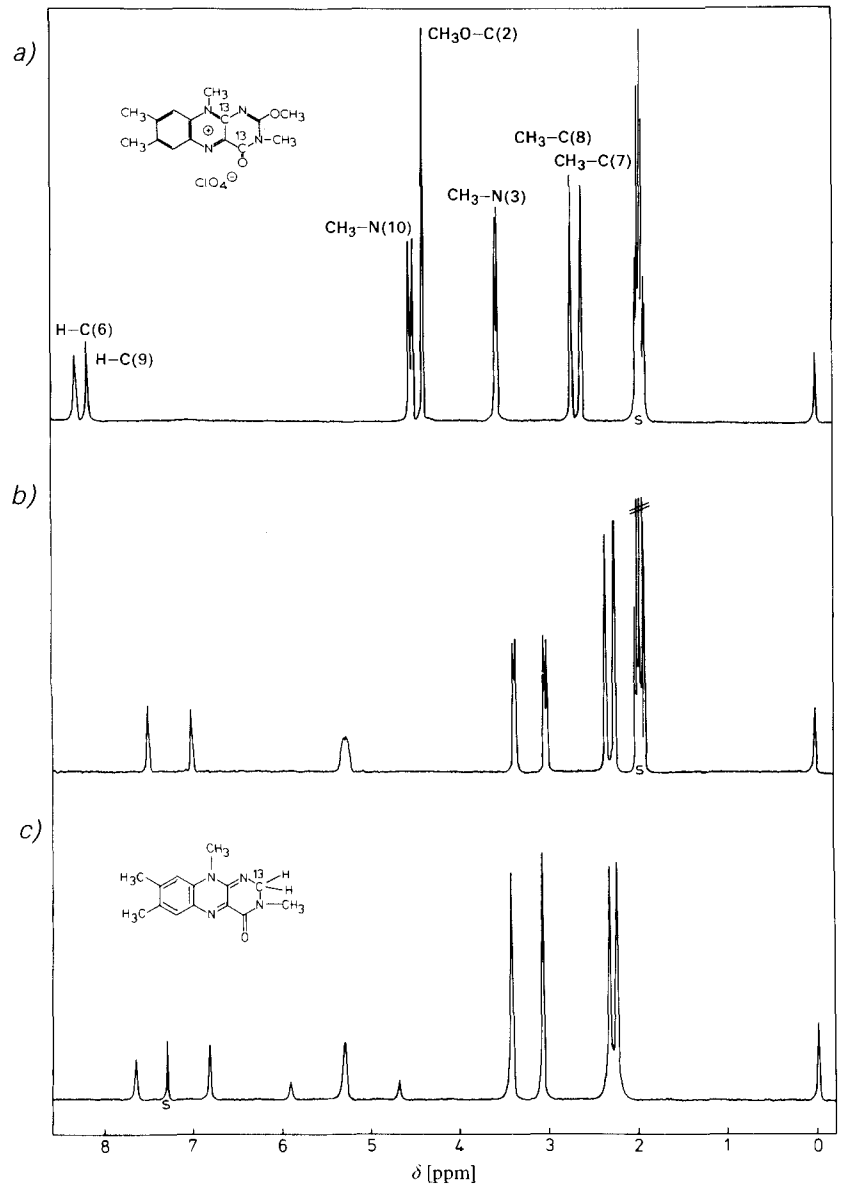


Fig. 2. *^{1}H-NMR spectra of* a) *[4,10a-$^{13}C_2$]-**6** and* b) *its borocyanohydride-reduction product [4,10a-$^{13}C_2$]-**7** in CD_3CN as well as* c) *[2-^{13}C]-**7** and **7*** (ratio 1:2) *in CD_3Cl*

^{13}C-enriched flavins which were obtained by small-scale syntheses (see *Fig. 2* for ^{1}H-NMR and *Table 2* for ^{13}C-NMR data).

In *Fig. 1a*, the ^{1}H-NMR spectrum of **6** in CD_3OD is shown. After addition of an excess $NaBH_3CN$ (solid material) to the CD_3OD solution, a ^{1}H-NMR spectrum was taken immediately (*Fig. 1b*) which exhibits one major product and at least one 'by-product'. The major 'by-product' represents most probably the '1,5-dihydro' deriva-

Table 1. *^{1}H-NMR Chemical Shifts* (in ppm) *of* **5**–**8**, **10**, *and* **13**[a])

	H–N(1)	H–C(2)	CH_3O–C(2)	CH_3–N(3)	H–C(6)	CH_3–C(7)	CH_3–C(8)	H–C(9)	CH_3–N(10)
5[b])	–	–	–	3.45	7.98	2.48	2.59	7.79	4.15
6[b])	–	–	4.41	3.57	8.32	2.61	2.73	8.18	4.53
7	–	5.30	–	3.06	7.42	2.25	2.35	6.99	3.41
8[c])	–	6.16	3.74	3.12	7.63	2.33	2.44	7.27	3.26
10	8.45[d])	5.18	–	3.16	8.01	2.47	2.56	7.79	3.83
13	–	8.75	–	3.68	8.33	2.63	2.75	8.26	4.65

[a]) Spectra were obtained in CD_3CN, except for **8** where the solvent was CD_3OD.
[b]) Taken from [20] where the assignments of H–C(6), H–C(9), CH_3–C(7), and CH_3–C(8) are discussed in detail.
[c]) Only observed in solution on reduction of **6** by $NaBH_3CN$.
[d]) Not present in the spectrum, when D_2O was added to the solution.

Table 2. *The Relevant ^{13}C-NMR Chemical Shifts* (in ppm) *of* **5**–**7**, **10**, *and* **13**[a])

Atom[b])	**5**[c])	**6**[d])	**7**	**10**	**13**
C(2)	156.7	160.7	74.6[e])	59.6	162.1[f])
C(4)	160.6	158.6	168.0	156.9	158.1
C(4a)	135.5	134.7	145.5	136.4	137.9
C(10a)	149.2	145.8	154.8	148.7	145.2

[a]) Spectra obtained in CD_3CN.
[b]) Enriched to at least 90 atom-%.
[c]) Taken from [21].
[d]) Taken from [22].
[e]) Appears as *t* in the undecoupled spectrum.
[f]) Appears as *d* in the undecoupled spectrum.

tive of **6**, *i.e.* **9** (*Scheme*). The spectrum of the main product exhibits resonances at 7.63, 7.27, 6.16, 3.74, 3.26, 3.12, 2.44, and 2.33 ppm (for the assignments see *Table 1*). Compared to the spectrum of **6**, all resonances are shifted upfield except for the additional line observed at 6.16 ppm, which represents 1H by integration. Therefore, the only chemical difference between the starting material and the main product present in *Fig. 1b* is 1 H-atom. The chemical shift of the latter led us to assign the spectrum of *Fig. 1b* to **8**. This assignment is supported by the subsequent spectra (*Fig. 1c* and *1d*) taken 20 min and 4 h, respectively, after addition of $NaBH_3CN$. *Fig. 1c* demonstrates that a second major product is formed with time. Most of the resonances in *Fig. 1c* are further upfield shifted as compared to those in *Fig. 1b*. There is one major difference between the two spectra; the resonance line at 5.30 ppm of the new product represents 2 protons indicating the formation of **7**. In *Fig. 1d*, **8** is no longer present, and **7** has already been considerably oxidized to **5**. The above data demonstrate that **8** is an intermediate in the reduction of **6** by borocyanohydride. The results show that **8** is rather easily formed, but it is also further transformed to **7** by borocyanohydride. The ^{1}H-NMR spectrum of purified **7** is identical with that assigned in *Fig. 1c* and *1d* to 7, fully supporting our interpretation.

In *Fig. 2a*, the ^{1}H-NMR spectrum of **6**, ^{13}C-enriched at positions 4 and 10a for easy identification of the resonance lines due to the CH_3N and CH_3O groups, is shown. The CH_3–N(10) and CH_3–N(3) resonance lines are split into *d* (^3J(C,H) ≈ 5 and 4 Hz, resp.). The ^{1}H-NMR spectra of the borocyanohydride-reduction products, namely [4,10a-$^{13}C_2$]-**7**, and a mixture of **7** and [2-^{13}C]-**7** in a ratio of 2:1, are shown in *Fig. 2b* and *2c*, respectively (the chemical shifts in *Fig. 2b* and *2c* differ slightly from each other and from those given in *Table 1* for **7** due to the different solvents used). The resonance line at 5.30 ppm in *Fig. 2b* is a *m* due to the two now almost equivalent ^3J(C,H) coupling constants (*ca.* 5 Hz), but it represents 2 protons as judged by integration. This already supports the correct assignment of the final borocyanohydride-reduction product to structure **7** and is consistent with the observed ^1J(C,H) of 130 Hz in *Fig. 2c*. The ^{13}C-NMR chemical shifts of the relevant compounds (*Table 2*) show that reduction of the N(1)=C(2) bond of **6** leads to an upfield shift of the C(2) resonance line by *ca.* 95 ppm, showing that this atom changed hybridization from sp^2 to sp^3, as expected.

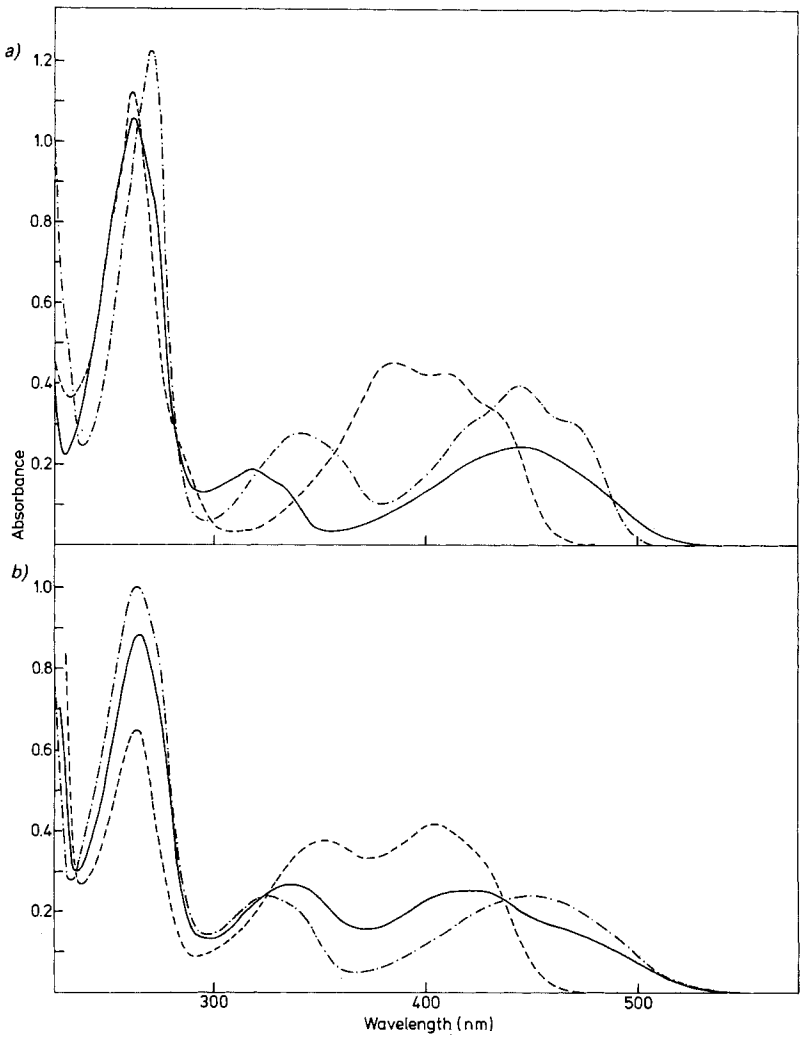


Fig. 3. *UV/VIS spectra of 25* μM *solutions of* a) **5** (–·–·–), **6** (– – –), *and* **7** (——) *in MeCN and of* b) **7** *in aqueous solutions at pH 0* (– – –), *pH 4.9* (——), *and pH 7.0* (–·–·–)

The UV/VIS characteristics of **5**–**7** are compared in *Fig. 3a*. The spectral properties of **7** are very similar to those of **5**, except that the second maximum in **7** is hypsochromically shifted by *ca.* 30 nm (*Table 3*). The pH-dependence of the spectrum of **7** is shown in *Fig. 3b*. From the pH-dependent changes at 400 nm, the ionization constant of 4.5 was calculated which is much higher than that of **5** ($pK_a = 0.18$ [12]). Elimination of the carbonyl group at position 2 in flavin renders the flavin nucleus more basic at N(1).

In previous papers [11] [13], we have shown that strictly monovalent C(2)-substituted flavins exhibit a characteristic, higher basicity (pK_a between 3.7 and 5.5) than the parent compound **5**. It is now obvious that also divalent C(2)-substituted flavins show a similar

Table 3. *UV/VIS Spectral Data of* **5**–**7**, **10**, *and* **13**

	Solvent	λ_{max} [nm] (ε[lmol^{-1} cm^{-1}])	F_{max} [nm][a])	Q[b])
5[c])	MeCN	444(12300), 340 (8800), 269(38600), 221(31200)	503	0.47
	pH 7	444(12400), 366(10000), 265(42200), –	525	0.25
6[c])	MeCN	410(12400), 386(13000), 261(32900), –	488	0.15
7	MeCN	447 (7500), 312 (5800), 275(32900), –	552	0.03
	pH 7	452 (7600), 325 (7200), 261(30300), 216(30300)	*ca.* 590	*ca.* 0.004
10	pH 0	404(12700), 353(11500), 263(20000), 221(39400)	488	*ca.* 0.007
	MeCN	394(12800), 350(9600), –, –	–	–
13	MeCN	401(17400), –, –, –		

[a]) F_{max} = Fluorescence emission maximum.
[b]) Quantum yield of fluorescence, absolute values.
[c]) Taken from [14].

behaviour. It is interesting to note that reduction of the 4-carbonyl group in **5** leads to a smaller increase in basicity [3] than that in position 2. This observation is in agreement with the notion that the 4-carbonyl group is less conjugated with the π-electron system of the isoalloxazine ring than the 2-carbonyl group.

In contrast to the UV/VIS, the fluorescence emission properties of **7** are quite different from those of **5**, except for its salt form **10** which shows the expected emission maximum at *ca.* 490 nm [14] (*Table 3*). The fluorescence emission maximum of **7** appears at longer wavelength as that of **5**. The relatively large *Stockes* shift observed in **7** and the broadened emission band suggest that the fluorophore possesses a strained configuration. The rather low quantum yield of **7** and **10** suggests a very efficient radiationless loss of excitation energy in these molecules. The excitation spectra are matching the light-absorption spectra indicating that the observed fluorescence emission is originating from the molecules in question. As mentioned above, the ionization constant of **7** is 4.5. Investigating the pH dependence of the fluorescence emission spectrum of **7**, it was found that, *e.g.* at pH 4.6, not the expected composed spectrum of **7** and **10** was observed, but solely the spectrum of **7**. The spectrum of pure **10** was only obtained at pH < 2. This indicates that **7** possesses a lower basicity in the excited singlet state than in the ground state. According to *Förster* [15], the excited-state pK_a (= pK_a^*) can be calculated from absorption and emission spectra. A value of *ca.* −1 was thus obtained for the pK_a^* of **7**. In analogy, it is likely that also **5** exhibits a lower ionization constant in the excited singlet state, but this cannot be determined, due to the already low basicity of **5** in the ground state [16].

Using $S_2O_4^{2-}$, **7** can be easily reduced to the corresponding 1,2,2,5-tetrahydro derivative **11**. The reduction is fully reversible in the presence of air and is analogous to the one of **5** [16]. Photoreduction of **7** in the presence of EDTA leads, in contrast to **5** [2], to photodestruction rather than photoreduction of **7**.

An almost quantitative yield of the cationic radical **12** (λ_{max} = 490 nm) was obtained from **7** in 2N AcOH under conditions used to produce the cationic semiquinone of **5** [17], but the neutral semiquinone could not be obtained, probably due to a more favored disproportionation reaction at neutral pH than observed for **5** [18].

The salt form **10** but not **7** can be converted photochemically to **13.** The spectral changes of this conversion are shown in *Fig. 4*. During the course of the photochemical

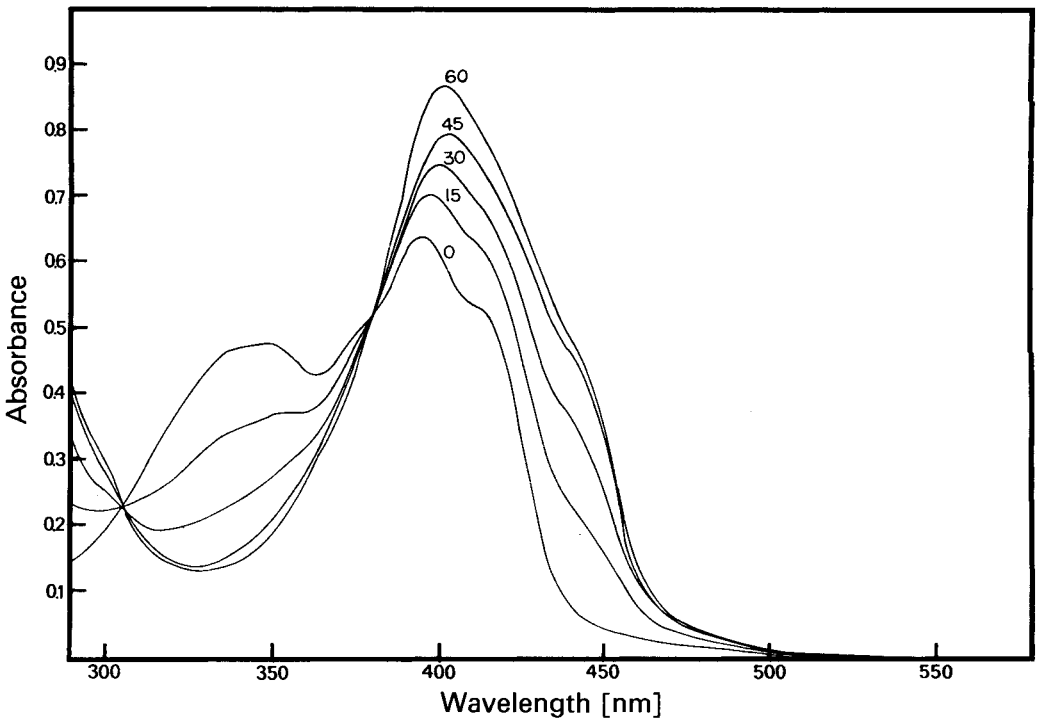


Fig. 4. *UV/VIS spectral changes observed on photochemical conversion of a 50* μM *MeCN solution of* **10** *into* **13**. The numbers refer to the irradiation time in min.

reaction, the maximum at 346 nm disappears, and the maximum at 394 nm shifts to 401 nm along with a hyperchromic effect. On TLC, two photoproducts are observed, the minor product being identified as **5**. The structure of the major photoproduct **13** is elucidated by its ^{1}H- and ^{13}C-NMR data (see *Table 1* and *2*). The conversion of **10** into **13** represents the type of photodehydrogenation which has not been observed for flavin compounds hitherto.

Comparison of the ^{1}H-NMR data of **10** and **13** reveals that the signal of H–N(1) of **10** is absent in the spectrum of **13**, and the resonance of H–C(2) is shifted from 5.18 to 8.75 ppm. This downfield shift (1-H signal) is consistent with the conversion **10**→**13**. This conclusion is fully confirmed by the ^{13}C-NMR spectrum where the C(2) resonance of **13** is shifted downfield by *ca.* 100 ppm in comparison to that of **10**, indicating a change in hybridization from sp^3 to sp^2. The ^{1}H- and ^{13}C-NMR chemical shifts of **6** and **13** are very similar, reflecting the resemblance of the two structures.

The observation that only **10** but not **7** can be converted to **13** lies in the fact that **13** is not stable in neutral and alkaline solutions, undergoing probably a rearrangement similar to that described by *Dudley* and *Hemmerich* [19] for other flavinium salts. The initial step in this reaction is the addition of an OH ion to C(10a) of the flavin nucleus followed by ring opening. On the other hand, photochemical treatment of **7** at pH > 7 or in MeOH yields **5**.

Experimental Part

General. All solvents used were of reagent grade or, for spectroscopic experiments, of spectroscopic grade (from *Merck*, Darmstadt). Irradiations: A 150-W medium-pressure Hg lamp was used for solns. of **10**; monochromatic 365-nm light was obtained using a narrow band filter; solns. of **10** in MeCN were irradiated by a collimated light beam; prep. photoreaction with a *Hanau-Q-150* lamp in an immersion-type photoreactor. TLC: silica gel *1B2* precoated plates *(Bakerflex)*; eluent MeCN. M.p.: electrothermal melting-point apparatus; uncorrected. UV/VIS spectra: *Cary-1650* spectrophotometer. Fluorescence emission and excitation spectra: *Aminco-SPF-500* spectrofluorometer; data uncorrected for possible background contributions; quantum-yield determinations using 3-methyllumiflavin as reference (see [14]). Ionization constant for **7**: spectrophotometrically determined at 23° and calculated from graphs of the differences of absorbance at 400 nm (pH 0.0–10.0); ionic strength = 0.1 for various buffer solns.; ^{1}H- and ^{13}C-NMR spectra: *Bruker-CXP-300* spectrometer; 300 MHz for ^{1}H, 75.6 MHz for ^{13}C; all spectra were acquired in the *Fourier*-transform mode using 5-mm tubes; sample solns. 1–10 mM in CD_3CN or CD_3OD (*Merck AG*, Darmstadt) at 26°; for ^{1}H-NMR, spectral width 1000 or 1500 Hz, acquisition time 4 or 2.66 s; pulse width 20 μs without pulse delay, TMS as internal standard (= 0 ppm); for ^{13}C-NMR, ^{1}H-noise decoupling conditions except where otherwise stated, spectral width 5000 Hz, acquisition time 0.8 s, pulse width 10 μs, pulse delay 1.0 s, dioxan as internal standard, data reported rel. to TMS (dioxan – TMS = 67.84 ppm). MS: *Jeol-JMS-100* spectrometer.

2-O,3,7,8,10-Pentamethylisoalloxazin-10-ium perchlorate[4]) (**6**·ClO_4^-) was described previously [20]. The ^{13}C-enriched analogs of **6** were prepared from the corresponding 3,7,8,10-tetramethylisoalloxazine as described in [21].

2,10-Dihydro-3,7,8,10-tetramethylbenzo[g]pteridin-4(3H)-one (**7**). To a suspension of 0.2 g of **6** in 75 ml of MeOH, 0.3 g of $NaBH_3CN$ were added and shaken for a few min, until a clear soln. was formed. This soln. was kept, protected from light, for 24 h at r.t. (yellow→dark brown). TLC: very small spot of **6** (typical greenish fluorescence); fast-moving spot of **5**, and red-brown main spot close to the origin (orange fluorescence). The solvent was evaporated at 30°. To the residue, 3 ml of MeOH were added followed by 50 ml of 1M H_2SO_4. This mixture was extrated with 3 × 50 ml of CH_2Cl_2 to remove **5** (depending on the amount of **5** formed, additional extractions may be necessary). The pH of the aq. soln. was then brought to 4 to 5 by addition of solid Na_2CO_3. After extraction with CH_2Cl_2 (3 × 30 ml), the org. phase was dried (Na_2SO_4), and evaporated at 30° and the residue dissolved in 3 ml of CH_2Cl_2. To this soln., 9 ml of Et_2O were added and the amorphous precipitate formed removed by filtration. The clear soln. was allowed to stand overnight at −20°: brownish-orange crystals. Evaporation of the mother liquor and repeating the crystallization procedure gave another crop of crystals. Total yield: 65 mg (*ca.* 50%) of anal. pure (by TLC) **7**. M.p.: dec. at 220–230°, darkening at *ca.* 150° without any apparent change of the crystals. MS: 256. Crystals of **7** unstable towards air within weeks, the salt of **7** (= **10**) can be stored as crystals for several months without any noticeable deterioration.

1,2,3,4-Tetrahydro-3,7,8,10-tetramethyl-4-oxobenzo[g]pteridin-10-ium Perchlorate (**10**·ClO_4^-). By gentle warming, 50 mg of **7** were dissolved in a minimum volume of MeCN. After dilution with an equal volume of 2M $HClO_4$ and after 12 h standing at 4°, the pale yellow crystals were filtered off: 65 mg (*ca.* 95%). M.p. 170° (dec.). Anal. calc. for $C_{14}H_{17}ClN_4O_5 \cdot 2H_2O$ (392.80): C 42.80, H 4.88, Cl 9.03, N 14.27, O 28.51; found: C 43.00, H 5.00, Cl 9.4, N 14.3, O 27.9.

3,4-Dihydro-3,7,8,10-tetramethyl-4-oxobenzo[g]pteridin-10-ium Perchlorate (**13**·ClO_4^-). A 100 μM soln. of **10** was irradiated with a *Hanau-Q-150* lamp in the photoreactor (monitoring spectrometrically at 401 nm and by TLC). At the end of the reaction, 2 products were observed by TLC. The trace component was identified as **5**. The major product was isolated in the following way: 3 l of a 100 μM photolysis soln. were evaporated at 30°. The residue was dissolved in 50 ml of H_2O by gentle warming and the resulting soln. concentrated to 20 ml under reduced pressure at 40°. On standing overnight at 6°, brown crystals precipitated: 60 mg (*ca.* 60%) of **13**·ClO_4^-. MS: 255.

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