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CHEMILUMINESCENCE—III

THE MECHANISM OF THE CHEMILUMINESCENT AUTOXIDATION OF 7-HYDROXY-6,7-DIHYDROLUMIFLAVIN AND SOME RELATED PTERIDINES

R. ADDINK and W. BERENDS

Biochemical and Biophysical Laboratory of the University of Technology, Julianalaan 67, 2628 BC, Delft, The Netherlands

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Abstract—In the conversion of **1** to **2**, a nonoxidative and a subsequent oxidative phase were distinguished. In the first phase the formation of the intermediates **3**, **4** and **5** was established. In the second phase the formation of a dioxetane is postulated as the immediate precursor in the light giving step. The autoxidative chemiluminescence appeared to be a general feature of 8-substituted pteridines bearing a Me group at the 7-position. The chemiluminescence spectra and their quantum yields were determined.

In previous publications[1,2] we described the chemiluminescent (CL) autoxidation in aqueous solution of 7-hydroxy-6,7-dihydrolumiflavin **1** to the corresponding 8-oxo compound **2** (Scheme 1). Evidence was provided that **2** was formed in the excited state.[2]

This investigation was extended to the autoxidation of 8-substituted lumazines and pterins bearing a methyl group at the 7-position. It appeared, that the autoxidative removal of their 7-Me group, in DMF with t-BuOK, was accompanied by CL too.

In this paper we report a study on the mechanism of the CL autoxidation in this series of compounds.

RESULTS AND DISCUSSION

The CL autoxidation of **1** occurred in alkaline medium. The most efficient production of light took place in the range pH 8–9. The yield of compound **2** did not vary (*ca* 50%) in this range, but declined rapidly at higher pH values. Above pH 11 compound **2** decomposed.

The course of the CL at pH 8.7 is given in Fig. 1 (curves c_1 and c_3). It was a slow reaction that lasted several days. Comparison of curve c_1 with c_3 showed that the time, at which the CL reached its maximum, was dependent on the buffer concentration.

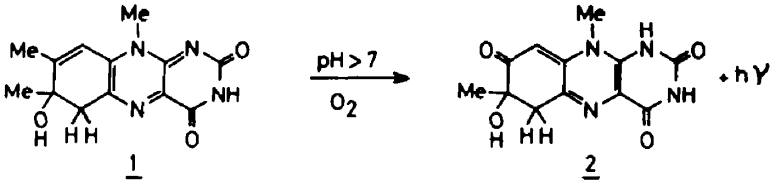
Repetitive recordings of the UV spectra during this reaction are given in Figs. 2 and 3. The changes at two selected wavelengths are summarized in Fig. 1. At pH 8.7 the spectra showed the disappearance of the absorption maximum at 445 nm of the starting compound **1** (Fig. 1, a_1, a_4; Fig. 2A) and the formation of a new absorption maximum at 344 nm (Fig. 1, a_2, a_5; Fig. 2A). The E 344 reached a maximum value and then decreased slowly again, while at the same time a new maximum at 418 nm (Fig. 2B), due to compound **2**, was formed.

Comparison of curve a_2 with curve a_5 showed that the formation of the intermediate 344, as well as the CL, was dependent on buffer concentration too. However, it is apparent that the intermediate 344 is not the direct precursor of the CL reaction, as the CL reached its maximum before E 344 became maximal.

As the CL is a direct consequence of a reaction with oxygen, the question arose whether this intermediate would be formed also in the absence of oxygen.

In an anaerobic solution at pH 8.7 initially the same spectral changes were observed as under aerobic conditions. The E 445 decreased in the same manner, but the course of E 344 was different. It reached a higher value (Fig. 1, a_3, a_6) and did not decrease. Subsequent addition of oxygen resulted in CL (Fig. 1, c_2, c_4) and a concomitant decrease of E 344. Compared to curves c_1 and c_3, the CL reached a higher value at a shorter time.

From this it follows that two intermediates were accumulated in the anaerobic reaction mixture: a precursor for the CL reaction and, in equilibrium with it, the intermediate 344.



1 2

Scheme 1.

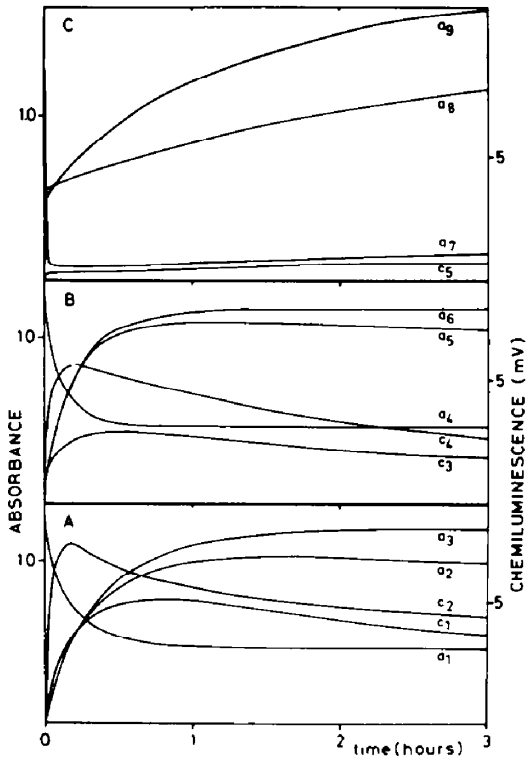


Fig. 1. Course of absorbance (E 445 and E 344) and chemiluminescence at 25° of a $6.7 \cdot 10^{-5}$ M solution of **1** in: A, 0.05M phosphate pH 8.7; a_1, E 445 aerobic as well as anaerobic; c_1, CL, t(max) 48 min; c_2, CL after anaerobic accumulation, t(max) 11 min; a_2, E 344 aerobic, t(max) 104 min, E(max) 1.03; a_3, E 344 anaerobic, E(max) 1.20; B, 0.1M phosphate pH 8.7; c_3, CL, t(max) 29 min; c_4, CL after anaerobic accumulation, t(max) 13 min; a_4, E 445 aerobic as well as anaerobic; a_5, E 344 aerobic, t(max) 1 hr, E(max) 1.10; a_6, E 344 anaerobic, E(max) 1.17; C. aerobic, 0.1N KOH; c_5, Cl, t(max) 3 hr; a_7, E 445; a_8, E 344, t(max) 7 hr, E(max) 1.30; a_9, E 344 in 0.05 M NaOMe in MeOH, t(max) 10 hr, E(max) 2.0.

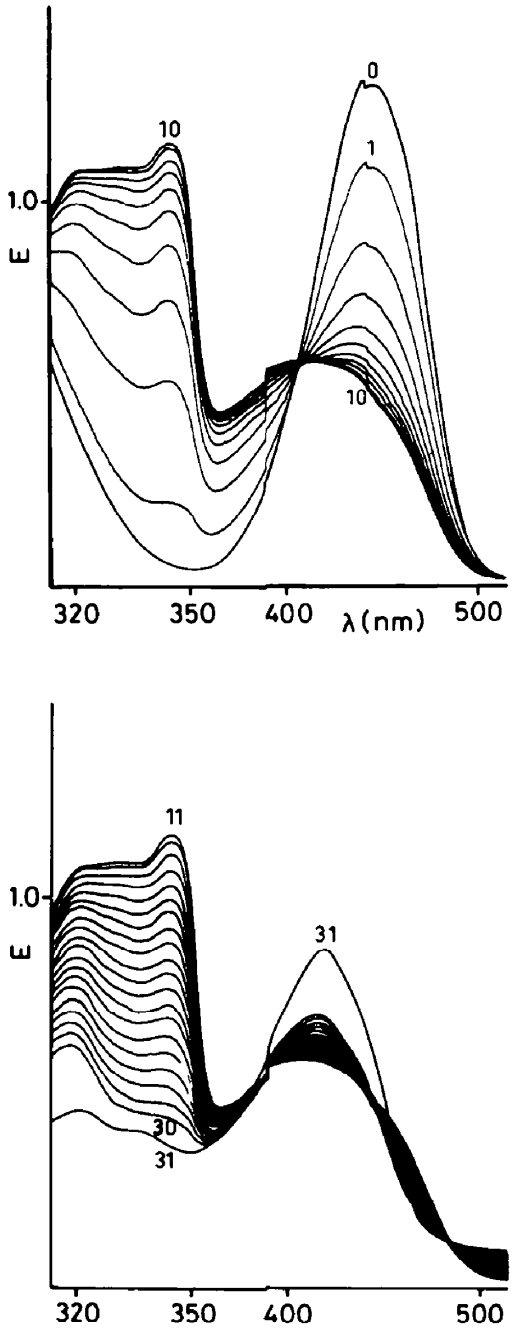


Fig. 2. Repetitive scan (one scan took 175 sec) at 25° of a $6.7 \cdot 10^{-5}$ M solution of **1** in water (0), and 0.1M phosphate pH 8.7 (1–31):
A, Spectra were started at 310 nm at 0.5 min(1) and every 3.5 min up to 25 min; 32; and 39 min(10) after addition of buffer;
B, Continuation: spectra were started at 49 min(11) and every 30 min up to 10,19 hr (30), and after 24 hr (31).

Careful acidification of the anaerobic reaction mixture after 130 min led to the quantitative recovery of the starting material **1**, according to the UV absorption spectrum. This indicates that, either a reaction of **1** with the solvent took place, or that tautomers of **1** were formed. The dependence on buffer concentration, indicating that the formation of the intermediates was subject to general base catalysis, is in accordance with this supposition.

In 0.1 N KOH a much faster disappearance of the E 445 (Fig. 1, a_7; Fig. 3A) occurred than at pH 8.7. The rise of E 344 took place in two stages. After an initial burst, it was built up slowly (Fig. 1, a_8; Fig. 3B). This means that in the initial burst, the E 344 is composed of the absorbances of at least two intermediates.

The CL of this solution was very low (Fig. 1, c_5). The total amount of light was only about 20% of that obtained at pH 8.7. It reached its maximum before E 344 became maximal.

From these observations it follows that in this medium three intermediates were formed and observed separately. In order of formation: (1) An intermediate formed immediately upon addition of base to **1**. Its formation was shown by the fast disappearance of E 445 and the initial fast rise of E 344. Further it is characterized by maxima at about 320 and 380 nm (Fig. 3A, spectrum 5). (2) The precursor for the CL reaction. (3) The intermediate with the 344 nm absorption maximum.

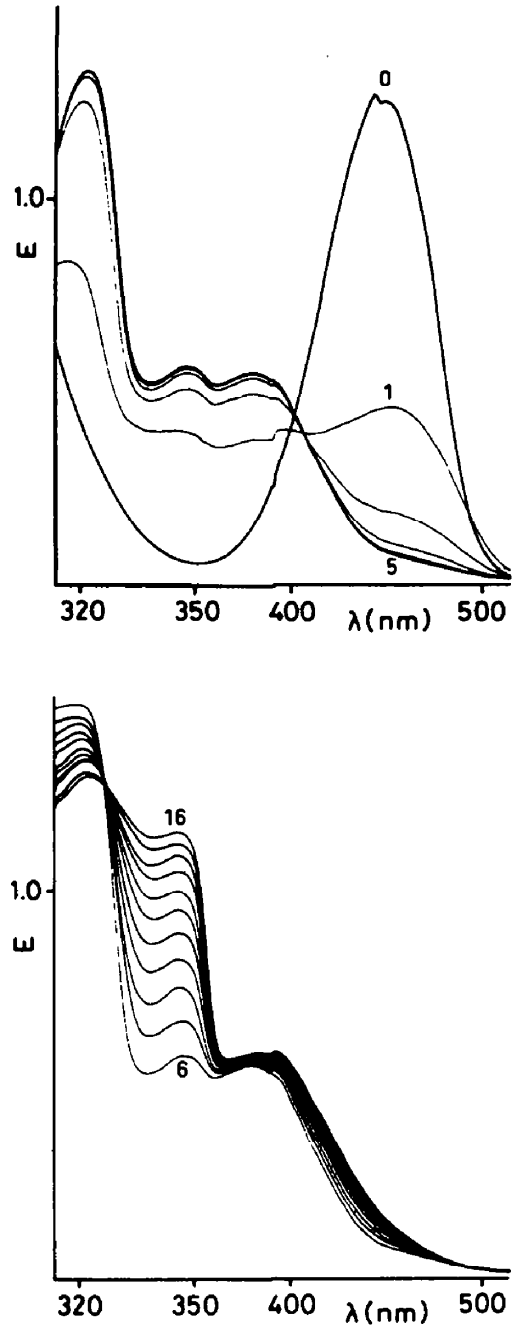


Fig. 3. Repetitive scan (one scan took 37 sec) at 25° of a $6.7 \cdot 10^{-5}$ M solution of **1** in water (0), and in 0.1N KOH(1–16): A, Spectra were started at 310 nm at 10 sec(1); 52; 95; 138; and 181 sec(5) after addition of KOH; B, Continuation: spectra were started at 5 min(6) and every 20 min up to 205 min(16). Then the solution was acidified.

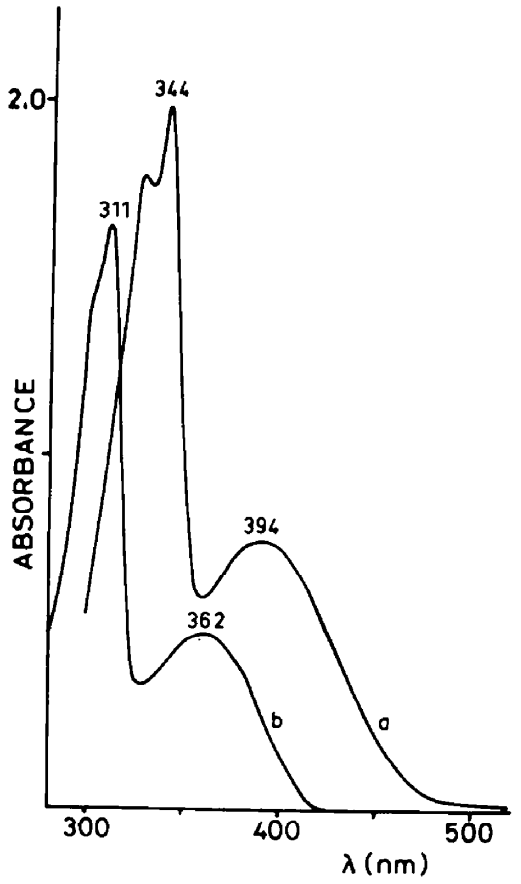


Fig. 4. (a) spectrum of a $6.7 \cdot 10^{-5}$ M solution of **5**[a] (R=Me) in 0.05M NaOMe in MeOH, obtained 10 h after dissolving **1**. (b) spectrum of a $6.7 \cdot 10^{-5}$ M solution of **9** (R=H) in 0.05M NaOMe in MeOH, obtained immediately after dissolving **8** (R=H).

When after 205 min (Fig. 3B) the solution was acidified, again the spectrum of **1** was quantitatively restored, indicating that no appreciable reaction with oxygen had taken place yet.

The influence of solvent was studied by carrying out the reaction in methanol with methoxide as a base. At low methoxide concentration (10^{-4}–$5 \cdot 10^{-4}$ M), CL and spectral changes similar to those given in Fig. 2 for the aqueous solution were observed. This means that the three intermediates mentioned above were also formed in methanol.

At relative high methoxide concentration (0.05M) UV spectral changes similar to those described for 0.1N KOH in water occurred. However, no CL was observed. The intermediate 344 again was built up slowly (Fig. 1, a_9), but after reaching its maximum, much higher than in aqueous solution, it did not decrease for at least 24 hr. (Decreasing the excess of methoxide, by adding formic acid for example, again resulted in CL and a concomitant decrease of E 344). Apparently, compound **1** was converted quantitatively into the intermediate 344. From the final spectrum (Fig. 4) it appeared that the molar absorbance of this intermediate was about 30,000 in methanol at 344 nm. The molar absorbance in 0.1N KOH and phosphate buffer, obtained by diluting the methanolic solution, was the same at 344 nm.

From this it was calculated that at pH 8.7 under anaerobic conditions (Fig. 1, a_3, a_6), at the highest about 60% of the intermediate 344 was formed.

The course of these reactions was also monitored by NMR. On adding methoxide to a suspension of **1** in methanol in an NMR tube, a solution was formed during a short time. Two absorptions at high field (1.13

and 1.32 ppm) of about equal intensity were then observed. These absorptions were attributed to the 7- and 8-Me groups of the OMe adduct **3** (R=Me), which contrary to the Me groups in **1**, had attained similar surroundings by attack of methanol at C_8. A precipitate was rapidly formed, which was studied further. The UV spectrum of this precipitate was identical to that of intermediate 344. This compound appeared to be a sodium salt to which structure **5**[a] was assigned on account of the following arguments:

(1) The IR spectrum showed the presence of only one CO absorption at 1625 cm^{-1}. From comparison with model compounds this was ascribed to the 4-CO band.

(2) The most notable features of the NMR spectrum in DMSO are: the presence of only one three proton absorption at high field (1.16 ppm) from the 7-Me group, the presence of two weakly coupled one proton absorptions at 4.65 and 4.92 ppm ascribed to the 8-methylene group, and a three proton absorption of a methoxy group at 3.17 ppm. This resonance did not seem to originate from methanol of crystallization. It could not be removed by drying in vacuum up to 100°. At higher temperature the compound decomposed.

In water similar reactions were observed by NMR. Addition of one equivalent of NaOH to **1** resulted in the formation of **3** (R=H), followed by a slow conversion into **5** (R=H).

The intermediates formed prior to the reaction with oxygen are summarized in Scheme 2: addition of a base to a solution of **1**, shifted the equilibrium between **1** and **3** and its anionic species **1**[a] and **3**[a] to the left. The formation of this adduct **3** was indicated by the disappearance of the visible absorption, and the appearance of two high field absorptions in NMR. The subsequent formation of **5**, **5**[a] was revealed by the appearance of a UV absorption maximum at 344 nm and two weakly coupled one proton absorptions in NMR. The third intermediate **4**, **4**[a], formed prior to **5**, is the precursor for the CL reaction. Compounds **3** and **5** act as storage intermediates from which the CL reaction, via **4**[a], is fed slowly.

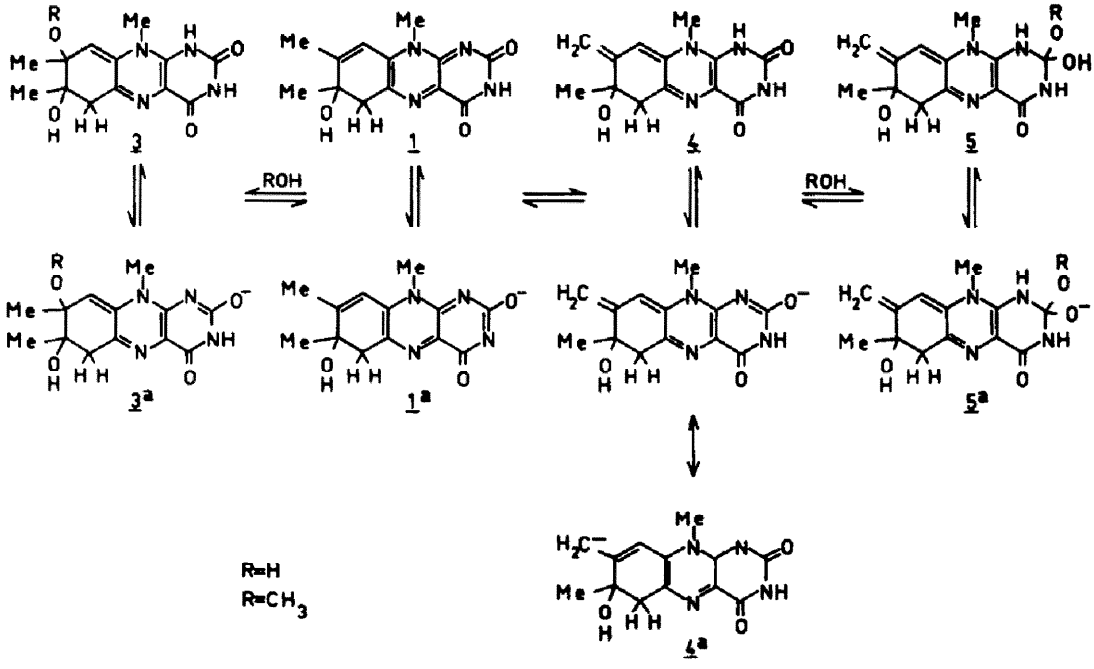
The formation of the 8-oxo compound **2** in the excited state[2] as the final product of the CL reaction, is reason to postulate a dioxetane as the immediate precursor in the light giving step. This indicates that as a second carbonyl compound formaldehyde had to be formed. This was found indeed (56% in aqueous solution and 82% with methanol as a solvent). The proposed route for the formation of the dioxetane **7** is given in Scheme 3. Oxidation of the anion **4**[a] is supposed to give the peroxymethylene anion **6**. An intramolecular nucleophilic ringclosure to the dioxetane **7** might then take place, as the 8-position is electrophilic.

Autoxidation of related pteridines

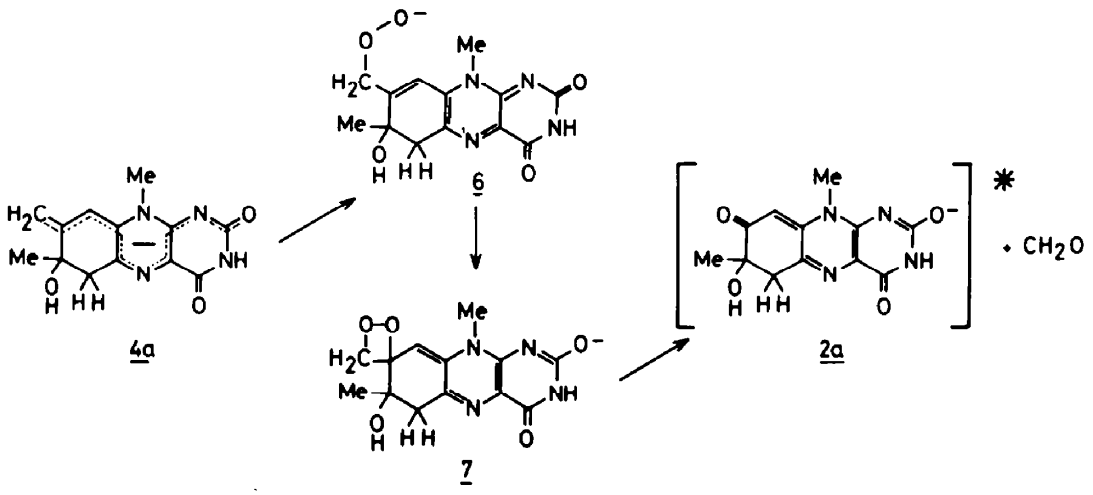
When 8-substituted-6,7-dimethyllumazines or -pterins are treated with aqueous alkali, the following reactions occur: Hydration at the 7-position.[3,4] Formation of a 7-methylene group.[5,6] Oxidation by oxygen, giving a 7-oxopteridine.[7]

These reactions which are similar to those shown by compound **1**, gave us the idea to investigate these pteridines for autoxidative CL. Although in aqueous alkaline medium autoxidation to the 7-oxopteridines did occur,[7] no CL was observed. In methanol with methoxide no autoxidation occurred and hence no CL was obtained.

In a polar waterfree solvent, like DMF or DMSO, a reasonably efficient, but slow CL reaction was observed upon addition of Et_3N or t-BuOK as a base.



Scheme 2.

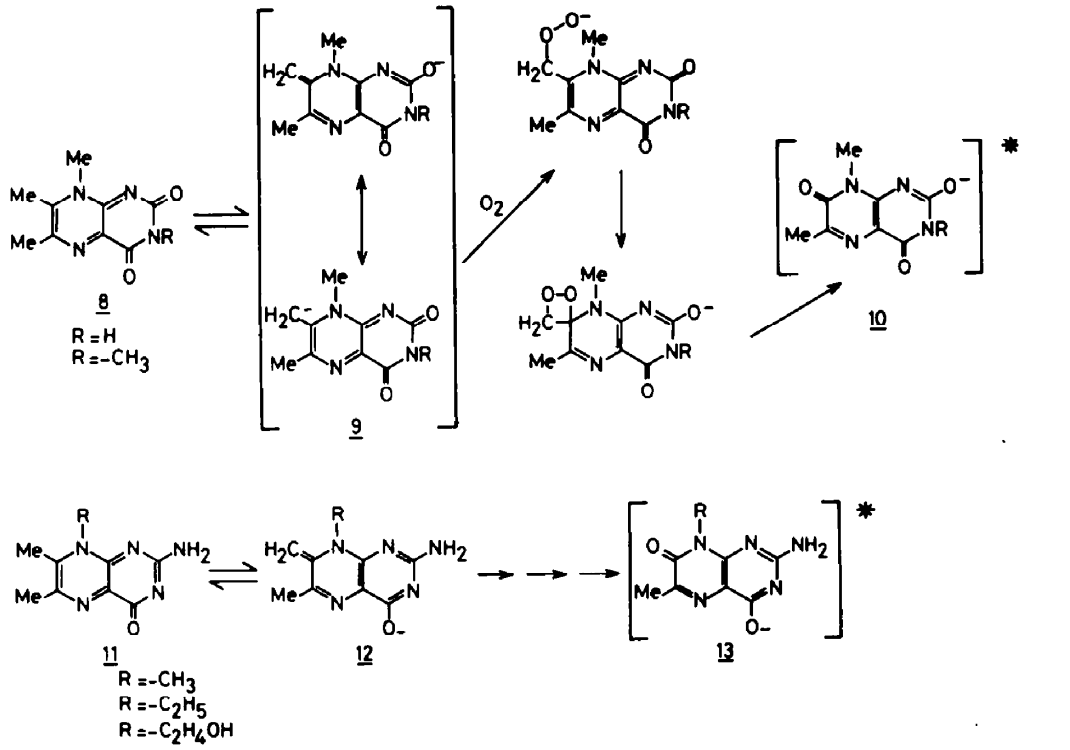


Scheme 3.

The results found in DMF/t-BuOK are presented here.

In Scheme 4 the proposed mechanism for the CL autoxidation of the lumazines **8** and pterins **11** is given. In the first phase of the reaction, addition of t-BuOK led to the fast formation of the anions **9** and **12**. UV absorption spectra taken within a few minutes after addition of base allready gave the final spectra of **9** and **12** (Table 1). The compounds **9** (R=H, Me) and **12** (R=Me) were isolated as their sodium salts by treatment of **8** respectively **11** with methoxide in methanol. According to NMR a methylene group was present in these compounds.

In Fig. 4 the UV absorption spectra of **9** (R=H) and **5**[a] (R=Me) in methanol/methoxide are given. Compared with **9** the spectrum of **5**[a], due to extended conjugation, is shifted to longer wavelength, but the



Scheme 4.

Table 1. UV absorption spectra in 10^{-3} M t-BuOK in DMF

Starting compound	λ_{max} (ε)
8 (R=H)	305 sh(17,800); 314(20,400); 372(11,000)
8 (R=Me)	304 sh(16,700); 312(19,500); 372(11,000)
11 (R=Me)	302 (16,600); 313(19,000); 375(8,200)
11 (R=Et)	304 (16,200); 315(18,900); 379(7,600)
11 (R=C_2H_4OH) [a]	303 (12,900); 314(14,400); 379(6,000)

[a]The competitive intramolecular attack of the β-hydroxy group on the 7-position is cause of the lower absorbances.

Table 2

Chemiluminescence

Compound[a]	Medium	ϕ_{es}	CL_{max}[b]	Fl_{exc}[c]	Yield[d]
1	H_2O, pH 8.7	$2.4 \cdot 10^{-3}$	480	417	0.50
1	MeOH/NaOMe[e]	$1.2 \cdot 10^{-2}$	485	417	0.62
Lumazines					
8 (R=H)[8]	DMF/t-BuOK[f]	$9.2 \cdot 10^{-3}$	438	362	0.50
8 (R=Me)[3]	"	$8.7 \cdot 10^{-3}$	439	362	0.63
Pterines.HCl					
11 (R=Me)[7]	"	$1.2 \cdot 10^{-2}$	441	366	0.44
11 (R=Et)	"	$6.4 \cdot 10^{-3}$	442	365	0.45
11 (R=C_2H_4OH)[9]	"	$3.9 \cdot 10^{-4}$	444	368	0.37

Fluorescence

Compound	Medium	ϕ_f	λ_{exc}[g]	Fl_{max}[b]	Fl_{max}[h]	Fl_{exc}[i]
2	H_2O, pH 8.7	0.75	417	479	486	417
2	MeOH/NaOMe[e]	0.71	417	485	489	417
Lumazines						
10 (R=H)[10]	DMF/t-BuOK[f]	0.79	363	438	420	362
10 (R=Me)[10]	"	0.80	363	438	420	362
Pterines						
13 (R=Me)[9, 11]	"	0.87	369	441	426	366
13 (R=Et)	"	0.82	369	443	430	365
13 (R=C_2H_4OH)[9]	"	0.83	369	444	438	368

[a]Concentration in CL reaction $2\text{–}3 \cdot 10^{-5}$ M.
[b]30 nm bandwidth, uncorrected, see Fig. 5.
[c]3.3 nm bandwidth; spent reaction mixtures.
[d]maximum yields of oxo compounds.
[e][NaOMe] = *ca* $5 \cdot 10^{-4}$ M
[f][t-BuOK] = *ca* 10^{-3} M.
[g]wavelength used for ϕ_f determination, 3.3 nm bandwidth.
[h]30 nm bandwidth, corrected.
[i]3.3 nm bandwidth.

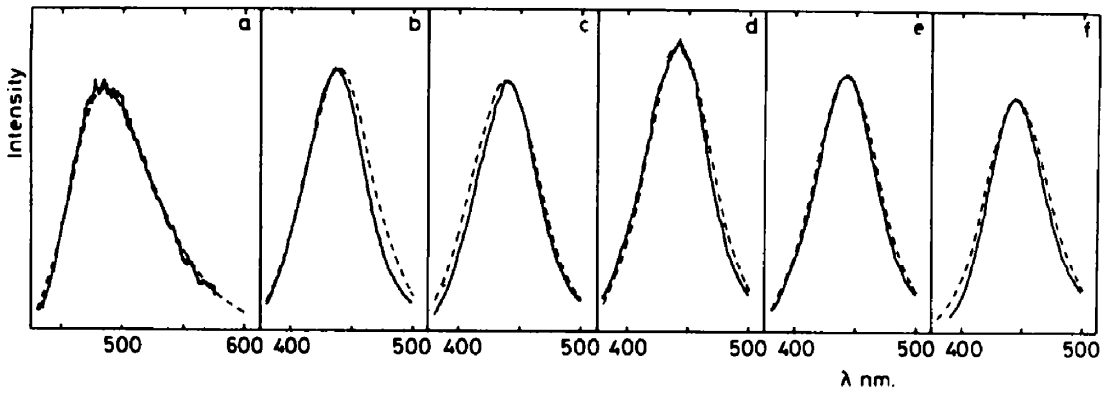


Fig. 5. Solid curves: CL spectra of a, **1** in MeOH/NaOMe; in DMF/t-BuOK of b, **8** (R=H); c, **8** (R=Me); d, **11** (R=Me); e, **11** (R=Et); f. **11** ($R=C_2H_4OH$). Dotted curves: fluorescence spectra of the spent reaction mixtures, identical with those of the authentic 7-oxo compounds.

general shape is very similar. Comparison with the spectra given in Table 1 shows that in DMF/t-BuOK also the 7-methylene compounds were formed.

In the second phase, autoxidation of **9** and **12** finally led to the formation of a 7-oxolumazine **10** or a 7-oxopterin **13** in the excited state.

The CL spectra matched the fluorescence spectra of the spent reaction mixtures and those of the authentic 7-oxo compounds (Fig. 5; Table 2). Also the fluorescence excitation spectra of the spent reaction mixtures were identical with those of the authentic 7-oxo compounds. This proves that the 7-oxo compounds were formed in the excited state.

CONCLUSION

The formation of the adduct **3** and compounds **4** and **5** (Scheme 2) upon treatment of the dihydroflavin with base under anaerobic conditions, strongly supports the proposed formation of a dioxetane as an intermediate in the CL reaction as given in Scheme 3.

The general CL autoxidation of the pteridine derivatives, proceeding via similar intermediates (**9** and **12**), further strengthens the validity of this mechanism.

EXPERIMENTAL

PMR spectra were recorded on a Varian A 60 or a Varian EM 360L spectrometer with TMS as an internal standard. The UV spectra given in Figs 2 and 3 were recorded on a Perkin–Elmer 402, the UV spectrum of Fig. 4 was recorded on a Perkin–Elmer 555. For quantum yield measurements, extinctions were determined on a Beckman UV 5260 or a Perkin–Elmer 555.

Chemiluminescence emission and fluorescence emission and excitation spectra were recorded on a spectrofluorimeter build in this laboratory. The relative spectral sensitivity was obtained using a calibrated tungsten lamp. Excitation spectra were corrected automatically by a Rhodamine B quantum counter.

The CL reaction was carried out in a flow system in which the reactants (appropriate solns of pteridin and base) were mixed 1:1 by a peristaltic pump. The mixture was led to a flow cell and the CL spectrum was recorded with a bandpass of 30 nm. The total light output was monitored by a second photomultiplier.

The total light produced in a CL reaction was monitored by a photo-multiplier RCA 5819, which viewed the bottom of a LSC vial containing 4 ml of a reacting soln, held at a temp of 25°.

The relative spectral senitivity of the RCA 5819 was obtained by placing it at the sample position of the spectrofluorimeter and irradiating it, after sufficient attenuation, with light isolated from a 1600 W Xenon arc with a double monochromator with a bandpass of 6.6 nm. The response of the PM was corrected for the lamp output, which was measured by a Rhodamine B quantum counter.

The CL yield ($\phi_{cl} = \phi_l . \phi_r . \phi_{ex}$) was obtained by integrating the area from the light output curve of the CL reactions. This was compared to the output of a liquid light standard.[12] (A mixture of 0.9 ml dry DMSO and 0.1 ml t-BuOH saturated with t-BuOK was injected into 1 ml of a luminol solution in dry DMSO in front of the RCA 5819 photomultiplier).

In order to make a meaningful comparison, the relative sensitivity of the PM to the different CL emission spectra was determined by a method given by Weber and Teale.[13]

The reaction yield (ϕ_r) was determined from the fluorescence of the spent reaction mixtures with an Eppendorf Digitalphotometer 6115.

The fluorescence yields (ϕ_f) were determined in relation to the fluorescence of quinine in 1N H_2SO_4 at 25°. Quinine · H_2SO_4 · H_2O was obtained from Aldrich and used without purification. Corrections were made for differences in refractive index. Care was taken that the absorbance of all solutions was below 0.1.

Demas and Crosby[14] recommend for quinine a $\phi_f = 0.546$ in 1N H_2SO_4 at 25° and 365 nm excitation. However, the absorption curve of quinine is rather steep at 365 nm, with an incline of *ca* 5% per nm. Small inaccuracies in wavelength from the spectrophotometer, as well as from the spectrofluorimeter and errors in the extinction determination caused by the bandwidth used (3.3 nm), might easily introduce errors up to 10%. As ϕ_f is constant upon excitation in the region 345–376 nm,[14] excitation in the absorption maximum at 347 nm, where these errors are eliminated for the greater part, ought to give the same ϕ_f. However, the ϕ_f we find by 347 nm excitation, lied about 10% below the value obtained by 365 nm excitation. Apparently errors are made indeed.

Therefore we decided to use, and recommend using as reference a $\phi_f = 0.546$ for quinine in 1N H_2SO_4 at 25 , and excitation in the absorption maximum.

Using this reference we found for 3-aminophtalimide a $\phi_f = 0.60$ in ethanol at 25° and 385 nm excitation, in agreement with literature.[14]

Reactions of 7-hydroxy-6,7-dihydrolumiflavin **1**

In NaOH/H_2O *and* NaOD/D_2O. (a) An NMR spectrum was taken of a suspension of **1** (0.02 g) in D_2O (0.5 ml): δ = 1.45 (C_7–Me); 2.32 (C_8–Me); 3.35 (C_6–H_2); 4.05 (N_{10}–Me); 6.90 (C_9–H). Addition of 40% NaOD (10 μl), resulted in a soln with an NMR spectrum of a mixture of **3** (R=H) and **5** (R=H): δ = 1.20 (C_8–Me, **3**); 1.35 (C_7–Me, **3**, **5**); 2.63 (C_6–H_2, **5**); 2.78 (C_6–H_2, **3**); 3.03 (N_{10}–Me, **3**); 3.08 (N_{10}–Me, **5**); 4.92 and 5.03 (C_8=CH_2, **5**); 5.40 (C_9–H, **5**). In the course of several hr the absorptions of **3** disappeared from the spectrum, while those of **5** became stronger. The final NMR spectrum was: δ = 1.35 (s, 3, C_7–Me); 2.61 (s, 2, C_6–H_2 3.08 (s, 3, N_{10}–Me); 4.90 and 5.02 (2s, 2, C_8=CH_2); 5.38 (s, 0.5, C_9–H). The resonance assigned to the C_9–H had only an intensity of 0.5 H. Acidification of the soln with 6N DCl in D_2O (30 μl), followed by heating led to lumiflavin (0.015 g, 84%). An NMR spectrum of this lumiflavin in CF_3COOD showed that in the 8-Me group one proton had exchanged against deuterium, and that the C_9–H had exchanged for 50% against deuterium. (Direct treatment of **1** with deuterated acid gave lumiflavin in which the C_9–H had not exchanged[1]).

(b) To a suspension of **1** (0.292 g) in deaerated water (3 ml) a soln of NaOH (3 g) in water (3 ml) was added. A warm, dark yellow soln was formed. Crystallization immediately occurred. After cooling, the ppt was filtered off, thoroughly washed with MeOH and dried over P_2O_5, yield 0.18 g. NMR in D_2O gave a spectrum identical to the one described above for the mixture of **3** (R=H) and **5** (R=H). NMR (d_6-DMSO), signals assigned to **3** (R=H): δ = 1.03 (C_8–Me); 1.22 (C_7–Me); 2.68 (C_6–H_2); 3.02 (N_{10}–Me); 4.38 (C_9–H); 9.27 (N_3–H, exchangeable) signals assigned to **5** (R=H): δ = 1.17 (C_7–Me); 2.5 (C_6–H_2, masked by DMSO); 3.12 (N_{10}–Me); 4.67 and 4.92 (C_8=CH_2); 5.23 (C_9–H); 9.33 (N_3–H, exchangeable). The absorptions at 4.67 and 4.92 ppm were not sharp. A decoupling experiment showed that they were coupled.

In MeOH/NaOMe. (a) Compound **1** (0.871 mg) was dissolved in MeOH (100 ml) containing NaOMe (0.05M). Recording UV spectra of this soln showed the conversion of **1** into **5** (R=Me). After 10 hr no further changes in the spectrum occurred: λ_{max}, (ε): 257 (23,500); 333 (27,100); 344 (30,400); 394 (11,000). This spectrum was stable for another 24 hours, then it began to decline slowly (*ca* 1% per day).

In the same manner **5** (R=Me) was allowed to accumulate from a more concentrated soln of **1** (0.0287 g in 100 ml 0.1M NaOMe in MeOH). Then it was diluted 60 times with 0.05M NaOMe in MeOH, giving the same spectrum as described above. It was also diluted 60 times with 0.1N KOH and with 0.1M phosphate buffer pH 9.5. These spectra were nearly identical, λ_{max} (ε, 0.1N KOH) (ε, pH 9.5): 254 (22,300) (22,900); 334 sh (27,800) (25,400); 344 (30,300) (31,100); 394 (10,000) (10,300). The spectra were measured immediately after the dilution was made. No changes occurred during the first few min. After a longer time the extinctions in the aqueous solns decreased, while in MeOH the spectra were stable.

(b) To a suspension of **1** (0.292 g), dried over P_2O_5, in deaerated MeOH (12 ml), a soln of 3M NaOMe in MeOH (0.67 ml) was added. A dark brown-green soln was obtained, from which crystals slowly appeared. After standing under N_2 for a few days, the dark green crystals were collected, washed with MeOH and dried over P_2O_5. Yield 0.257 g, sodium salt of **5**[a] (R=Me), $C_{14}H_{17}N_4O_4Na$ = 328.3. The NMR spectra in D_2O and d_6-DMSO were identical with the spectra of **5** (R=H), except for the additional presence of a three proton absorption at 3.33 ppm (D_2O) and 3.17 ppm (d_6-DMSO) of the OMe group. The UV spectrum was identical with that described under (a).

Sodium salt of 3,6,8-*trimethyl-7-methylenelumazine* **9** (R=Me). Compound **8** (R=Me) (0.05 g) was dissolved in MeOH (4 ml) and 2M NaOMe in MeOH (0.3 ml) was added. Slowly crystallization occurred. After standing overnight it was filtered off, washed with MeOH and dried in vacuum over P_2O_5, yield 0.036 g. NMR (d_6-DMSO): δ = 2.05 (s, 3, C_6–Me); 3.07 and 3.11 (2s, 6, N_3–Me and N_{10}–Me); 3.84 and 3.98 (2d, J = 1.5 Hz, 2, C_7=CH_2). In a similar manner we obtained:

Sodium salt of 6,8-*dimethyl-6-methylenelumazine* **9** (R=H). NMR (d_6-DMSO): δ = 2.00 (C_6–Me); 3.10 (N_{10}–Me); 3.78 and 3.93 (C_7=CH_2).

Sodium salt of 6,8-*dimethyl-7-methylenepterin* **12** (R=Me). NMR (d_{18}-HMPA, 100 MHz): δ = 2.06 (C_6–Me); 3.20 (N_{10}–Me); 3.92 and 4.02 (C_7=CH_2).

8-*Ethyl*-6,7-*dimethylpterin.hydrochloride* **11** (R=Et). 2-Amino-6-ethylamino-4-hydroxy-5-nitrosopyrimidine[15] (3.7 g) was hydrogenated with 10% Pd/C (0.4 g) at room temp in a mixture of AcOH (30 ml) and water (45 ml). After H_2 uptake had stopped, the catalyst was filtered off under N_2 and diacetyl (1.8 ml) was added to the colourless soln. After heating for 30 min at 60–70°, the green soln was neutralized with $KHCO_3$ and cooled to 0°. The ppt was collected, yield 2.94 g. For analysis 0.50 g was treated with conc HCl (0.4 ml) in EtOH (5 ml). Addition of ether gave a ppt, which was crystallized twice from EtOH, yield 0.31 g, m.p. > 300°(Found: C, 46.2; H, 5.5; N, 27.1. ($C_{10}H_{13}N_5O$.HCl (255.71). Calc. for: C, 46.97; H, 5.52; N, 27.39%). NMR (CF_3COOD): δ = 1.62 (t, J = 7 Hz, 3, N_{10}–Et); 2.83 and 3.00 (2s, 6, C_6–Me and C_7–Me); 4.94 (q, J = 7 Hz, 2, N_{10}–Et). After standing overnight the absorption at 3.00 ppm (C_7–Me) had nearly completely exchanged against deuterium.

8-*Ethyl*-6-*methyl*-7-*oxopterin* **13** (R=Et). To a suspension of 2-amino-6-ethylamino-4-hydroxy-5-nitropyrimidine (1.5 g), prepared in a similar fashion as the 6-methylamino derivative,[7] in water (40 ml), 2N KOH (30 ml) was added. The soln was heated on a steam bath and solid dithionite was added until a light yellow soln was obtained. The pH was adjusted to pH 10 with HCl and the soln was cooled. The light yellow 2,5-diamino-6-ethylamino-4-hydroxypyrimidine was collected (0.71 g) and taken up in 0.15N HCl (29 ml). After addition of Me-pyruvate (2 ml), the solution was refluxed during 1 hr. Upon cooling a ppt was formed (0.64 g), which was recrystallized thrice from water, yield 0.32 g, m.p. > 300°. Found: C, 45.6; H, 5.6; N, 29.6. (Calc. for: $C_9H_{11}N_5O_2.H_2O$ (239.23) C, 45.19; H, 5.48; N, 29.27%). NMR (CF_3COOD): δ = 1.40 (t, J = 7 Hz, 3, N_{10}–Et); 2.88 (s, 3, C_6–Me); 4.52 (q, J = 7 Hz, 2, N_{10}–Et).

Determination of formaldehyde

In water. Formaldehyde was determined as its adduct with dimedon.[16] This adduct is very insoluble in water. Addition of dimedon to the CL mixture however, did not give a ppt. Even addition of CH_2O prior to the reaction only gave a very small amount of adduct afterwards. Apparently, reaction products bound the CH_2O. Therefore we developed a method using ^{14}C labeled CH_2O, and measuring the dilution of the specific radioactivity. A soln of **1** (0.147 g) in 0.2 M NH_4HCO_3/NH_4OH buffer pH 8.5 (1 l) was allowed to autoxidize in the presence of $^{14}CH_2O$ (0.254 mmole). After 24 hr the yield of **2** was 30%. The reaction was stopped by adding conc H_2SO_4 (5 ml) and the water was distilled off at normal pressure. From blank experiments it appeared, that CH_2O was quantitatively obtained in the distillate in this manner. Dimedon (0.11 g) was added to the destillate and after several hr the precipitate was filtered off, washed with water and dried, yield 0.075 g. The specific radioactivity, determined in a LSC, had decreased with 25% in relation to the adduct formed from dimedon and "undiluted" $^{14}CH_2O$. From this it was calculated, that in the CL reaction 56% CH_2O was formed in relation to the amount of **2**.

In methanol. The following procedure was developed from the results of blank experiments: A soln of **1** (0.146 g), dried over P_2O_5, in 10^{-3} M NaOMe in MeOH (1 l) was allowed to autoxidize. After 9 days the yield of **2** was 43%. Dimedon (0.28 g) and HAc (0.06 ml) were added. After 24 hr, water (4 l) was added and after again 24 hr 1N NaOH (7 ml) was added. The mixture was now evaporated to a small volume and was filtered. Then 1N HAc (7 ml) was added and the ppt was

collected, yield 0.051 g, coloured material, adduct of CH_2O and dimedon, 82% in relation to the amount of **2** formed. It was taken up in water (200 ml) and 1N KOH (2 ml) and charcoal (0.2 g) were added. After stirring for 10 min the charcoal was filtered off and the filtrate was acidified with 1N HAc (2 ml). The ppt was collected and dried, yield 0.031 g, white crystals, m.p. 186–8° (lit.[16] 189°). This same purification procedure was carried out with authentic dimedon-CH_2O adduct (0.051 g) and yielded 0.031 g, m.p. 186–8°, mixed m.p. 186–8°. Both products were also identical according to IR.

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