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# A Novel Hydroxylation of Aromatics in a Flavin-Initiated Chain Reaction

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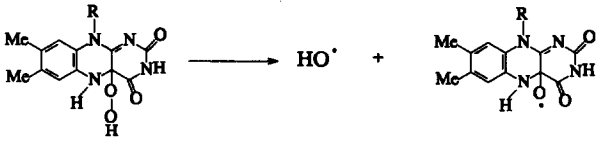
***Abstract:*** In aqueous acidic solutions, the 5-ethyl-3-methyllumiflavinium cation **5** was spontaneously transformed into the dihydroflavin pseudobase radical **6** and the flavosemiquinone **7**. In attacking an aromatic like phenylalanine, **6** was mainly reconverted to **5** which, ultimately, could lead to an anaerobic accumulation of **7** in yields of 95 ± 5%. In the immediate presence of $O_2$ and/or $H_2O_2$, **7** was rapidly and continuously reoxidized to **5** which, consequently, gave a continued production of **6** increasing the hydroxylating ability of the system. The oxidants also had a second distinct effect in converting an intermediate **X** in a chain reaction to further increase the yields of hydroxyphenylalanines. As illustrated by the results obtained with $O_2$ and $H_2O_2$ under comparable conditions, the efficiency of this chain reaction proved to be significantly influenced by the nature of the oxidant. These new findings imply that hydroxyl radicals arising in a homolysis of the O-O bond in a dihydroflavin hydroperoxide should not be taken for granted as the primary attacking species in the hydroxylation of an aromatic. From a practical point of view it is noticed that the hydroxylating ability of the new flavin / $H_2O_2$ system surpasses that of any other known, flavin-free chemical system.

## Introduction

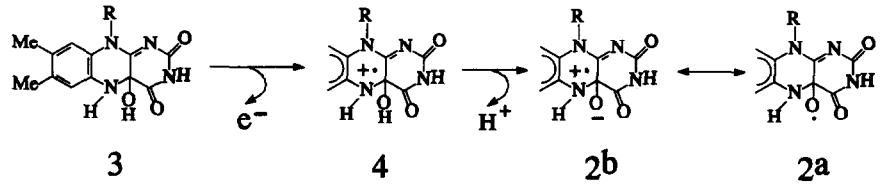
Hydroxyl and flavinoxy radicals **2a** are the products of homolysis of the O-O bond in a dihydroflavin hydroperoxide **1** (Eq. 1).



**1** **2a** (1)

Concerning the ability of a dihydroflavin hydroperoxide **1** to hydroxylate aromatic compounds, the flavinoxy radicals **2a** were proposed to be even more reactive than $HO^{\bullet}$ radicals in directly attacking the substrate.[1,2] In order to further substantiate this assumption, a method had to be developed to more specifically generate flavinoxy radicals **2a** without the simultaneous formation of hydroxylating $HO^{\bullet}$ radicals. A basic principle for this was sought[3] in the one-electron oxidation of a dihydroflavin pseudobase **3** ( Eq. 2) to give the pseudobase radical **4** chemically,[4] electrochemically,[5] and in a reaction with $N_3^{\bullet}$ radicals generated by pulse radiolysis.[6] The pseudobase radical **4** is the protonated form of the N-centered zwitterionic radical **2b** being in resonance[6] with the O- centered flavinoxy radical **2a**.

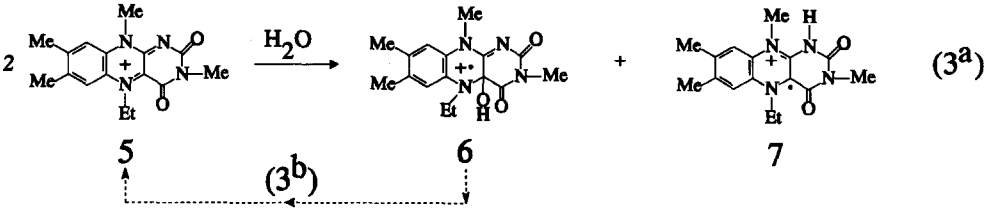
The chemical method is the simplest to apply. In a variety of solvents,[4] the 5-ethyl-3-methyllumi-



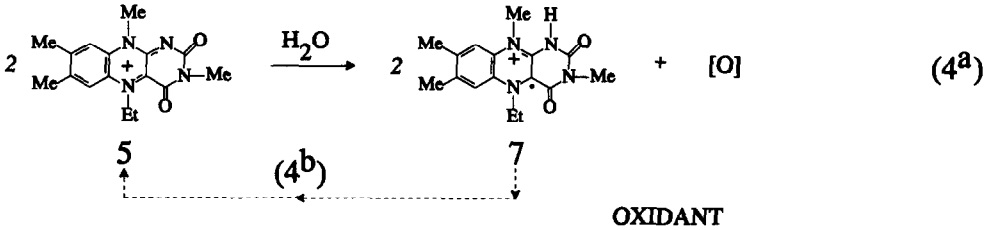

(2)

3 4 2b 2a

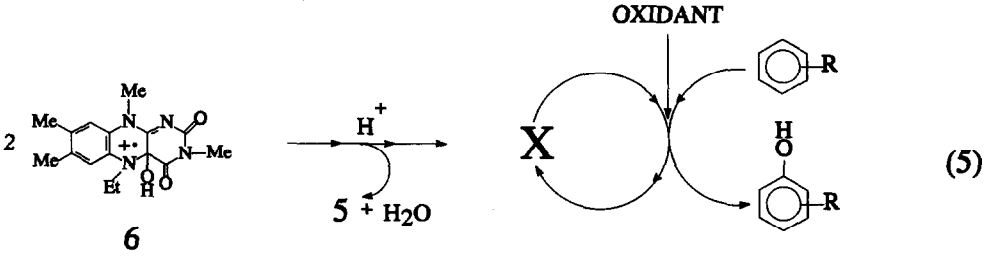
flavinium cation **5** was found to be transformed spontaneously to a dihydroflavin pseudobase radical (i.e. 5-ethyl-4$^a$-hydroxy-3-methyl-4$^a$,5-dihydrolumiflavin radical cation **6**) and the unprotonated and/or protonated flavosemiquinone (i.e. **7**; Eq. 3a). In aqueous, acidic environment,[3] due to a consecutive, spontaneous conversion of **6** → **5** (pathway 3b), the overall *anaerobic* process ultimately led to the accumulation of the protonated flavosemiquinone **7** in yields of 95 ± 5%, consistent with the stoichiometry reflected by Eq. 4a. Under $N_2$, in the presence of phenylalanine as a substrate, the "missing oxygen" **[O]** was partially accounted for by the formation of tyrosine and its *m*- and *o*-hydroxyphenylalanine isomers. The overall yields of 2 - 40%, depending on the acidity of the medium (0.05 - 6.0 N $H_2SO_4$), correspond to HOPhe/Fl ratios of 0.01 - 0.20 (the maximal theoretical HOPhe/Fl ratio is 0.5). These were the first results in strong support of the assumption on a direct attack of aromatics by flavin radical species.




5 (3$^b$) 6 7 (3$^a$)




5 (4$^b$) 7 (4$^a$)




6 (5)

Additionally, an unidentified intermediate **X** (Eq. 5) was accumulated in low yields consistent with the high yields of flavosemiquinone obtained. After replacing the $N_2$-atmosphere by air, **X** reacted in an oxidative chain reaction, without a further supply of the starting flavin, to considerably increase the yields of hydroxyphenylalanines (Eq. 5).

The present paper deals with the spontaneous formation and subsequent reactions of the flavin radicals **6** and **7** (Eq. 3a) in the presence of phenylalanine and in the *immediate* presence of $O_2$ and/or $H_2O_2$ to establish the occurrence of two different effects increasing the hydroxylating ability of the system.

## Results and Discussion

The presence of $O_2$ and/or $H_2O_2$ primarily caused a fast and continuous oxidation of **7** to **5** (pathway 4b) and, consequently, a continued transformation of **5** to the transient **6** (Eq. 3a) on account of which the hydroxylating ability of the system may already be expected to be higher than that of the anaerobic system in the absence of such oxidants.[3] Since the oxidation of **7** was relatively rapid, only the spectrum of **5** was observed, slowly changing due to an irreversible conversion of the starting flavin (Fig. 1). The spectral changes were used to monitor the process which was continued until **5** was no longer detected (cf. curve *h*). After the disappearance of **5**, the pH was adjusted to 7.0, catalase was added to destroy any $H_2O_2$ present, after which most products derived from **5** were removed by extraction with chloroform. The aqueous layer was subjected to HPLC analysis to give the results in HOPhe/Fl values (the totals of *p*-, *m*- and *o*-hydroxyphenylalanines per starting flavin) as summarized in Tables 1 and 2. The results in the Tables were corrected with the data from the HPLC analyses of the appropiate controls of flavin solutions containing all the components except phenylalanine and, solutions containing all the components

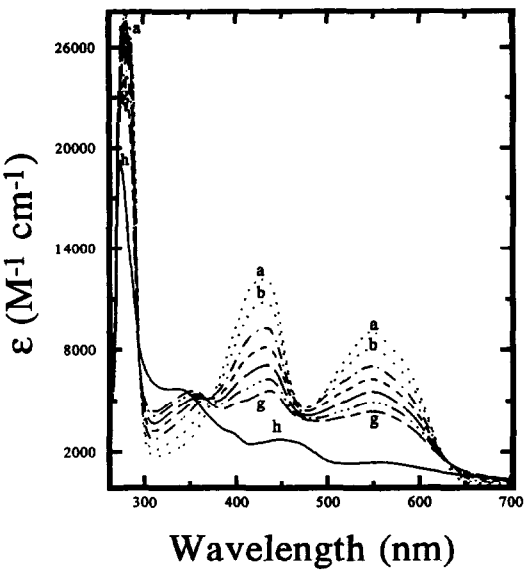



**Fig. 1.** Irreversible conversion of **5** (1 - 4 x $10^{-4}$ M) at 50 °C in 0.1 N $H_2SO_4$ containing phenylalanine (0.08 M) under air. The spectra were taken at reaction times of (**a**) 0 (**b**) 25 (**c**) 90 (**d**) 150 (**e**) 215 (**f**) 270 (**g**) 325 min; (**h**) 18 h. Isosbestic points at: 295 (**a** → **g**) and 375 nm (**a** → **c**). After 90 min, the isosbestic point at 375 nm was rather rapidly replaced by an isosbestic point at 368 nm (**e** → **g**).

except flavin **5**, subjected to the same treatments. Particularly upon increasing the temperature as in expts. 22 and 23 (Table 2), the flavin-free controls may show some formation of hydroxyphenylalanines arising in a reaction of phenylalanine with $HO^{\bullet}$ radicals formed in a homolysis of $H_2O_2$.

In the range of 12 - 6 N $H_2SO_4$, as compared to the anaerobic experiments in the absence of an additional oxidant,[3] the immediate presence of $O_2$ (expts. 1-2; Table 1) gave a modest, 2 - 3 fold increase in the overall yield of hydroxylation. An average of 10 fold increase was obtained, under comparable conditions, by the use of $H_2O_2$ under $N_2$ (expt. 13; Table 2).

In the range of 4 - 0.05 N $H_2SO_4$ (expts. 3 - 11; Table 1), the HOPhe/Fl values considerably increased with decreasing acidity of the medium, while the opposite (decreasing HOPhe/Fl values) was found under anaerobic conditions.[3] Therefore, the results of expts. 3 - 11 are ascribed, for the greater part, to a different effect of $O_2$, more particularly, as an oxidant in the chain reaction (Eq. 5).

The presence of $O_2$ affected the isomer distribution in stong acidic solutions. In 12 to 6 N $H_2SO_4$ (expts. 1 - 2), no net production of *m*-hydroxyphenylalanine was found as in solutions of lower acidity (expts. 3 - 12). A net consumption of *m*-hydroxyphenylalanine in 12 - 4 N $H_2SO_4$ was established to occur in control experiments containing *m*-hydroxyphenylalanine added before the start of the radical formation. Remarkably, no consumption of *m*-hydroxyphenylalanine was found in the same solution when air was replaced by $H_2O_2$ under $N_2$ (cf. expt. 13 *vs* 2). In the presence of $O_2$, no consumption of *m*-hydroxyphenylalanine occurred in

| **Expt.** (°C) | **Acidity** | **HO-Phe / Fl** | ***p* : *m* : *o* HO-Phe** | **Expt.** (°C) | **Acidity** | **HO-Phe / Fl** | ***p* : *m* : *o* HO-Phe** |
|---|---|---|---|---|---|---|---|
| ***1*** (20 °C) | 12 N | 0.46 | 45 : 0 : 55 | ***7*** (20 °C) | 0.1 N | 1.44 | 28 : 26 : 46 |
| ***2*** (20 °C) | 6 N | 0.29 | 39 : 0 : 61 | ***8*** (20 °C) | 0.05 N | 1.52 | 30 : 25 : 45 |
| ***3*** (20 °C) | 4 N | 0.44 | 34 : 7 : 59 | ***9*** (37 °C) | 0.1 N | 1.52 | 26 : 29 : 45 |
| ***4*** (20 °C) | 1 N | 0.95 | 28 : 20 : 52 | ***10*** (50 °C) | 0.1 N | 1.42 | 28 : 27 : 45 |
| ***5*** (20 °C) | 0.5 N | 1.20 | 29 : 23 : 48 | ***11*** (50 °C) | 0.05 N | 0.85 | 32 : 26 : 42 |
| ***6*** (20 °C) | 0.2 N | 1.19 | 29 : 24 : 47 | ***12*** (50 °C) | 0.1 N **(HCl)** | 0.99 | 31 : 24 : 45 |

**Table 1:** Formation of *p*, *m*- and *o*-hydroxyphenylalanine during the spontaneous transformation of the flavinium cation **5** (1 - 4 x $10^{-4}$M) to flavin radicals in the presence of phenylalanine (0.08 M) and ***air***, in non-stirred $H_2SO_4$ solutions (expts. 1 - 11). Expt. 12 was performed in non-stirred 0.1 N HCl.

The complete disappearance of **5** required: 5 days (expt.1); 3 weeks (expts. 2 - 8); 3 days (expt. 9); 18 h (expts. 10 - 12).

the range of 0.5 - 0.05 N $H_2SO_4$ (expts. 5 - 12) as was also reflected by the minor fluctuations in the isomer distributions. Maximal yields of hydroxylation (HOPhe/Fl= 1.42 - 1.52) were obtained in the range of 0.1 - 0.05 N $H_2SO_4$ (expts. 7 - 8). These values were about 150 times higher than the yields obtained under anaerobic conditions.[3] It is noted that the pH in the 0.1 N and 0.05 N solutions was rather high (1.8 and 2.7, respectively), due to the relatively high concentration of phenylalanine (0.08 M).

The complete disappearance of the starting flavin, upon exposing the solutions to air, was a rather slow process. However, an increase of the temperature from 20 to 50 °C considerably affected the rate of the process without seriously changing the HOPhe/Fl values and the isomer distribution in 0.1 N $H_2SO_4$ (cf. expts. 9 and *10 vs* 7). The spectral changes were monitored as exemplified for expt. 10 (Fig. 1). Two isosbestic points (at 295 and 375 nm, the latter changing to 368 nm) were observed for several hours, suggesting a rather straightforward destruction of **5**. Raising the temperature of a 0.05 N $H_2SO_4$ solution (expt. 11 *vs* 8) lowered the yield of hydroxylation whereas no significant changes in yields were detected in 0.1 N $H_2SO_4$ over 20 to 50 °C (expts. 7, 9 and 10). The yield of hydroxylation also decreased upon replacing the sulfate by the chloride anion (expts. 12 vs 10), confirming an anion effect on flavin intermediates as was already observed in earlier studies.[1b]

Replacing air by $H_2O_2$ (under nitrogen) to continuously recycle **7** to **5** gave a faster destruction of **5**. The processes were monitored by following the spectral changes which were closely similar to the ones depicted in Fig. 1.

| Expt. (°C) | Acidity | HO-Phe / Fl | *p* : *m* : *o* HO-Phe | Expt. (°C) | Acidity | HO-Phe / Fl | *p* : *m* : *o* HO-Phe |
|---|---|---|---|---|---|---|---|
| ***13*** (20 °C) | 6 N | 1.30 | 31 : 26 : 43 | ***20*** (20 °C) | 0.05 N | 6.03 | 27 : 30 : 43 |
| ***14*** (20 °C) | 4 N | 2.68 | 32 : 25 : 43 | ***21*** (30 °C) | 0.1 N | 14.62 | 24 : 27 : 47 |
| ***15*** (20 °C) | 2 N | 4.35 | 32 : 20 : 48 | ***22*** (37 °C) | 0.1 N | 14.69 | 27 : 29 : 44 |
| ***16*** (20 °C) | 1 N | 4.55 | 30 : 22 : 48 | ***23*** (37 °C)* | 0.1 N | 11.22 | 27 : 26 : 47 |
| ***17*** (20 °C) | 0.5 N | 6.47 | 28 : 24 : 48 | ***24*** (20 °C)* | 0.1 N | 9.56 | 27 : 28 : 45 |
| ***18*** (20 °C) | 0.2 N | 11.81 | 26 : 27 : 47 | ***25*** (20 °C)* | 0.05 N | 3.75 | 28 : 29 : 43 |
| ***19*** (20 °C) | 0.1 N | 15.08 | 26 : 29 : 45 | * $H_2O_2$+ air | | | |

**Table 2:** Formation of *p*, *m*- and *o*-hydroxyphenylalanine during the spontaneous transformation of the flavinium cation **5** (1 - 4 x $10^{-4}$ M) to flavin radicals in the presence of phenylalanine (0.08 M) and $H_2O_2$ (4.8 x $10^{-2}$ M) in non-stirred $H_2SO_4$ solutions, *all carried out under* $N_2$, with the exception of expts. 23, 24 and 25, *which were performed under air*.

The complete disappearance of **5** required: 7 - 12 days (expts. 13 - 20); 48 h (expt. 21); 24 h (expts. 22; 23); 4 - 5 days (expts. 24; 25).

The acidity of the solutions (cf expts 13 - 20; Table 2) had a great effect on the production of hydroxyphenylalanines, dramatically rising to a value of HO-Phe/Fl= 15.08 in 0.1 N $H_2SO_4$ (expt. 19). As with the experiments in Table 1, the yields of hydroxylation increased with decreasing acidity of the medium in contrast with the trend found under anaerobic conditions in the absence of an additional oxidant.[3] For the greater part, these results in Table 2 are to be ascribed to the effects of $H_2O_2$ and/or $HO^{\bullet}$ as oxidants in the chain reaction, much stronger than the effect of $O_2$ in experiments 5 - 12 of Table 1.

A raise of temperature from 20 °C to 37 °C in 0.1 N $H_2SO_4$ (expt. 21 and 22 *vs* 19), though considerably increasing the rate of the process, hardly affected the yield of hydroxylation and the isomer distribution. However, the additional presence of $O_2$ during the recycling lowered the yields of hydroxylation (expt. 23 *vs* 22; 24 *vs* 19; 25 *vs* 20). Such an inhibiting effect of $O_2$ was also observed in earlier studies.[1a-b]

The HOPhe/Fl values in Table 2 were determined immediately after the disappearance of **5**. It is emphazised that these data do not represent the optimal yields of hydroxylation. This was shown by subsequent analyses of a portion of the reaction solutions which, for that purpose, had not been treated with catalase. For example, the continued presence of $H_2O_2$ in the reaction solution of expt. 22 for two more days led to more hydroxyphenylalanines with the HOPhe/Fl value increasing from 14.69 to ~ 31. This is a 3000-fold increase as compared to the result of the anaerobic hydroxylation[3] in the absence of $H_2O_2$. Moreover, it is 60 times higher than the *theoretical* yield of HOPhe/Fl= 0.50. Although expressed as a HOPhe/Fl value, it should be kept in mind that the increased yield was obtained in the absence of **5** indicating that some accumulation of **X** had occurred; apparently, **X** had a longer life time than **5**.

In summary, in spite of a faster destruction of **5**, considerably higher yields of hydroxylation were found when $O_2$ was replaced by $H_2O_2$ (cf. expt. 22 in Table 2 *vs* expt. 9 in Table 1), clearly demonstrating an effect of the oxidant in the chain reaction (Eq. 5). Apparently, $H_2O_2$ or the derived $HO^{\bullet}$ is a far more effective oxidant than $O_2$. Particularly at higher temperature, $HO^{\bullet}$ radicals, arising in a homolysis of $H_2O_2$, could probably become more important as an oxidant in the chain reaction.

These new findings are in further support of our assumption on the role of intermediate **X**. Its possible formation in a disproportionation of **6** (Eq. 5) and its characterization as a dicationic flavin or rearranged derivative are subjects of continued studies. It is expected that further experiments on the purified compound may also reveal the optimal conditions to achieve aromatic hydroxylations.

## Conclusion

Studies on the spontaneous transformation of the flavinium cation **5** in aqueous solutions (Eq. 3a) to the dihydroflavin pseudobase radical **6** have been continued in order to show that the hydroxylating ability of such a system would increase in the immediate presence of an oxidant. The dihydroflavin pseudobase radical

**6**, which could directly attack an aromatic compound, was rapidly reconverted to **5** (pathway 3b). Ultimately, under anaerobic conditions in the absence of any additional oxidant, an almost quantitative accumulation of the flavosemiquinone **7** could be achieved (Eq. 4a).

The immediate presence of an oxidant during the formation of the flavin radicals **6** and **7** resulted in two, clearly distinguishable effects, in accomplishing:

(I) a continuous recycling of the flavosemiquinone **7** to the flavinium cation **5** and, subsequently, to the flavin pseudobase radical **6** increasing the hydroxylating ability of the system;

(II) a conversion of an intermediate **X** in a chain reaction (Eq. 5) further increasing the hydroxylating ability.

In comparing the oxidants $O_2$ and $H_2O_2$, we have demonstrated that the two effects are not necessarily linked to each other. Concerning the oxidative recycling of the flavosemiquinone **7** to the flavinium cation **5** (pathway 4b), $O_2$ proved to be less destructive than $H_2O_2$ or the derived $HO^{\bullet}$ radical. The use of $H_2O_2$, however, led to considerable higher yields of hydroxylation. The chain reaction could even be continued after the disappearance of **5** showing that some **X** had been accumulated.

Upon calculating the energetics of the cleavage of the O-O bond in a dihydroflavin hydroperoxide, in relation to monooxygenase activity, it has recently been concluded[7] "in sufficiently apolar enzyme pockets, homolysis of the O-O bond may become more favorable than other processes." There is a tendency to automatically assume that the $HO^{\bullet}$ radicals arising in such a homolysis (Eq. 1) would act as the primary attacking species.[8] The present findings, however, are in strong support of an early speculation[1] that flavinoxy radicals **2a** (Eq. 1) might fulfil such a primary role.

Apart from a basic implication as discussed above, our result may also be of significance from a more practical point of view. The yields of hydroxylation achieved with the flavin / $H_2O_2$ system were considerably higher than those obtained with any other known (flavin-free) hydroxylating systems in which, for example, $HO^{\bullet}$ radicals were generated by homolysis of $H_2O_2$ or by the use of Fenton's reagent.

## Experimental

**Instrumentation.** A Milton Roy, Spectronic 3000 Array absorption spectrophotometer was used to record the absorption spectra. Both anaerobic and aerobic experiments were carried out by using a special apparatus consisting of a 1-cm light path quartz cuvette fused to one or two compartments, provided with valves, allowing the reactants to be mixed at any moment, for example, in the anaerobic experiments after $N_2$ was flushed through for 1-3 h. The reaction volumes were 10 - 12 ml. $N_2$ was purified over a BASF R3-11 catalyst. HPLC was performed using a Waters μ Bondapak $^{TM}$ C18 (3.9 x 300 mm) column; solvents A/B (9:1); flow rate= 0.6-1.0 ml/min; UV detection at 275 nm, absorption mode. Solvent A= 0.05 M $CH_3COONH_4$

in $H_2O$, pH was adjusted to 4.9 using $CH_3COOH$; solvent B= 0.1 M $CH_3COONH_4$ in $H_2O$ (pH = 4.9) / $CH_3OH$ (1:4 v/v).

**Chemicals and Reagents.** 5-Ethyl-3-methyllumiflavinium perchlorate (**5**,$ClO_4^-$) was prepared as described in a previous paper.[3] Phenylalanine, tyrosine and 30% $H_2O_2$ were purchased from Aldrich Chemical Co. DL-*m*- and DL-*o*-Hydroxyphenylalanine and catalase were bought from Sigma.

D-Phenylalanine was preferred as a substrate because of the best purity in the HPLC-analyses.[3] Stock solutions (0.08 M) were prepared in 0.05 - 12 N acidic solutions. The reaction volumes varied from 10 to 12 ml. The starting flavin concentration was in the range of 1 - 4 x $10^{-4}$ M.

When the spectral changes had come to an end, $NaHCO_3$ was added to adjust the pH to 7.0 using $H_2O$ to dilute the reaction mixture. The dilution factor was kept as low as possible ($\leq$ 2.5). Most of the degradation products derived from **5** were removed by five extractions with 1-2 ml portions of $CHCl_3$ after which $N_2$ was blown through the aqueous layer to remove the $CHCl_3$ remnants. Samples of the aqueous solution were further diluted with water and subjected to HPLC analysis.

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