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THE OXIDATION OF ETHYL 1,2-DIHYDRO-2-NAPHTHOATE BY FLAVINS AND ITS STIMULATION BY LIGHT

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SUMMARY

1. Riboflavin, lumiflavin and 3-methyllumiflavin have been shown to oxidize ethyl 1,2-dihydronaphthoate to ethyl 2-naphthoate.

2. The reactions are stimulated by light. The second order rate constant of the riboflavin-catalyzed oxidation, in the dark, at 95° is 25 $l \cdot mole^{-1} \cdot min^{-1}$ while the same reaction in the light (15-W fluorescent tube at a distance of 30 cm), at 25°, has a rate constant of 540 $l \cdot mole^{-1} \cdot min^{-1}$.

3. Although somewhat complex, the pH dependency of the photooxidations parallels the ability of the flavins to fluoresce. At pH values greater than 7, riboflavin and lumiflavin lose their oxidizing ability while 3-methyllumiflavin is as effective at pH 11 as it is at pH 7.

4. The photooxidation is only weakly affected by changes in the ionic strength. Some ions were found to inhibit the reaction and most of these are paramagnetic.

5. The oxidation, in the dark, has a heat of activation equal to +14.6 kcal/mole while the photooxidation has one of −2.25 kcal/mole.

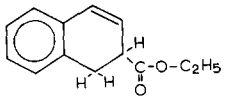
6. The other isomeric dihydronaphthoates were not oxidized, but some were isomerized.

7. This is the first report of a non-enzymic flavin-catalyzed oxidation of a carbon–carbon bond, α to a carbonyl functional group.

INTRODUCTION

Coenzymes containing riboflavin participate in a great variety of enzyme-catalyzed redox processes which have been well studied. However, the corresponding studies with non-enzymic model reactions have been limited to the oxidation of NADH[1,2], NADH analogs[3] and dihydrolipoic acid[4]. In addition to these reactions, which are stimulated by light[5,6], other compounds are oxidized only in the presence of light. Such compounds are amino acids or amines[7–12], carboxylic acids[12–15] and others[15]. Also, in the presence of light riboflavin will oxidize its own ribityl side-chain yielding flavins bearing shorter side-chains in the 9-position[16–21]. Most of these photocatalyzed reactions have little, if any, relationship to known biochemical reactions.

Among other biochemical reactions of flavin coenzymes is the oxidation of fatty acid acyl-CoA derivatives or succinic acid to α,β-unsaturated compounds. It was a desire to establish a model for this rather unique oxidation reaction which led us to a study of the oxidation of dihydronaphthoate esters. At first we studied simpler analogs such as the ethyl succinates and ethyl dihydrocinnamate. These were not oxidized but ethyl 1,2-dihydro-2-naphthoate (I) was oxidized by flavins slowly in the dark and very rapidly in light.



I

Recently, biological oxidations involving reduced benzenoid compounds have been investigated. The enzymic systems which oxidize dihydrobenzoic acid derivatives[22,23] are very analogous to the system reported here and it has been shown that a flavin is required for the oxidation of hexahydrobenzoyl-CoA to benzoic acid[24].

EXPERIMENTAL

Materials

The 2-naphthoic acid, obtained from Eastman Organic Chemicals, was recrystallized before use (m.p. 185–185.5°). The dihydronaphthoic acids were prepared by reduction of 2-naphthoic acid with sodium amalgam in basic aqueous solution[25]. The 1,4- and 1,2-dihydro acids were separated by fractional precipitation and were subsequently recrystallized from water. The 3,4-dihydro acid was prepared by heating the 1,2-dihydro acid with $Ba(OH)_2$ solution in a sealed tube at 140° for 10 h (m.p.: 3,4-dihydro, 117°; 1,4-dihydro, 160°; 1,2-dihydro, 101°). The spectral properties (λ_{max} and ε_M) of these compounds were identical to those reported by SCHRECKER *et al.*[26].

The ethyl esters of the dihydro-2-naphthoic acids were prepared using an excess of ethanol with dry HCl as a catalyst. (An attempt to prepare the ester of the 1,2-dihydro acid by the thionyl chloride method gave dark unidentifiable products.) The boiling points of the three esters were all 265–270° with much darkening. Values of 159–160, 163, and 152–153° (under 12 mm Hg) have been reported for the 3,4-dihydro, 1,4-dihydro, and 1,2-dihydro esters, respectively[27]. The spectra of these esters were nearly identical to the spectra of the respective acids in alcohol solution.

Riboflavin (U.S.P. readily soluble form), was obtained from Commercial Solvents Corp., dried and stored in the dark. FMN was supplied gratis by the Sigma Chemical Comp. Isoriboflavin obtained from the California Corp. for Biochemical Research was desiccated, and used without further purification. Lumiflavin, 2-lumiflavimine (2-deoxy-2-iminolumiflavin) and 3-methyllumiflavin were prepared by the method of HEMMERICH *et al.*[28]. Other flavins were prepared and characterized as described by SUELTER AND METZLER[3].

Methods

The studies on the effects of pH, ionic strength, solvent, and inorganic ions on the photochemical reaction were carried out with a pyrex tube of the following

dimensions: inside diameter, 1.81 cm; height, 15 cm; wall thickness, 0.119 cm. This tube was placed 30 cm from a 15-W Sylvania daylight type fluorescent tube with a white reflector. To standardize the procedure, the reaction was also conducted in a 1-cm silica cell which was similarly located.

For study of the effect of temperature on the light-induced reaction, the pyrex tube was immersed in a constant temperature bath. The fluorescent lamp was placed outside the glass bath container a few centimeters from the reaction tube.

During the course of the photochemical reactions, aliquots were removed from the reaction tube at intervals and the absorbance was measured at the appropriate wavelength. In all cases, a riboflavin blank was used to correct for the absorbance of riboflavin in the region employed. The extent of oxidation of 1,2-dihydronaphthoate was calculated from changes in the absorbance at 330 mμ which were confirmed at 260 mμ.

A typical reaction mixture consisted of 8 ml of 0.15 mM riboflavin solution, 16 ml of water, and 0.8 ml of 4 mM ethyl 1,2-dihydro-2-naphthoate. In the various experiments, the total volume remained constant with the amount of water being varied to compensate for the increase of the other components. All of the components, except the dihydronaphthoate, were placed in an aluminum foil-covered test tube. The dihydronaphthoate was placed in a glass cup suspended from the test tube stopper. (A rubber stopper boiled in NaOH solution and thoroughly washed.) After a minimum of 5 min, the test tube was inverted and the components were mixed. The test tube was removed from its aluminum foil cover and placed in the light beam. Aliquots were removed at given time intervals and were analyzed as outlined above.

Reactions were carried out in the dark at elevated temperatures in screw-top vials. The vials were wrapped with aluminum foil and black plastic tape to exclude light completely. Individual vials were taken out of the covered metal bath and plunged into ice water at various times. The reaction was followed by spectrophotometry as with the light-promoted reaction except that all manipulations were done in nearly complete darkness.

Complete spectra were traced by a Cary Model 14 recording spectrophotometer.

RESULTS AND DISCUSSION

Stoichiometry

In most experiments the reaction mixture was an aqueous solution, 0.125 mM in ethyl 1,2-dihydronaphthoate and 0.05 mM in flavin. We calculate that about 0.26 mM dissolved O_2 was present initially in the solution, the tubes of which were left open to air during the reaction. Since reduced flavins are rapidly and completely oxidized by air, we assumed that as dihydroflavins were produced they would be reoxidized by air immediately. This assumption is born out by spectrophotometric observation at 445 mμ. No decrease in the height of the flavin peak at this wavelength was observed during the reaction. When the spectrum of the reaction mixture was measured against that of a control solution containing riboflavin, but not ethyl dihydronaphthoate, a spectral change with time occurred as is shown in Fig. 1. This figure shows the change resulting from irradiation with a 15-W fluorescent lamp at a distance of 30 cm. The dihydronaphthoate spectrum is rapidly converted into one which closely approaches that of authentic ethyl 2-naphthoate and in which the

characteristic multiple peaks of the latter are faithfully displayed. An isosbestic point is observed at 275 mμ. In the absence of riboflavin, no change in the spectrum occurs during a comparable period of time.

A nearly identical but much slower change is observed in the dark. The isosbestic point is somewhat less sharp, probably indicating the occurrence of minor side reactions.

What we are reporting is, in fact, a photooxidation of ethyl 1,2-dihydro-2-naphthoate by O_2 with catalysis by riboflavin. Under anaerobic conditions, the riboflavin absorbance at 445 mμ decreases; however, we did not base our investigation upon this criterion because as shown in previous studies[5–21], there are many conditions which result in the loss of flavin absorbance. Also, it could be argued that the reaction is different in the presence or absence of O_2. Therefore, we elected to show that the riboflavin-dependent oxidation was an unambiguous conversion to a dehydrogenated product. The requirement of flavins for the occurrence of the reaction, the known ease of oxidation of dihydroflavins by O_2, and the kinetic data strongly suggest that the rate-limiting step is the hydrogen transfer from the dihydronaphthoate to the flavin. If the reoxidation of reduced flavin were rate-limiting, we would expect to find a rate increase if the solution was oxygenated during irradiation. This was not observed, but there was, rather, a slight inhibition.

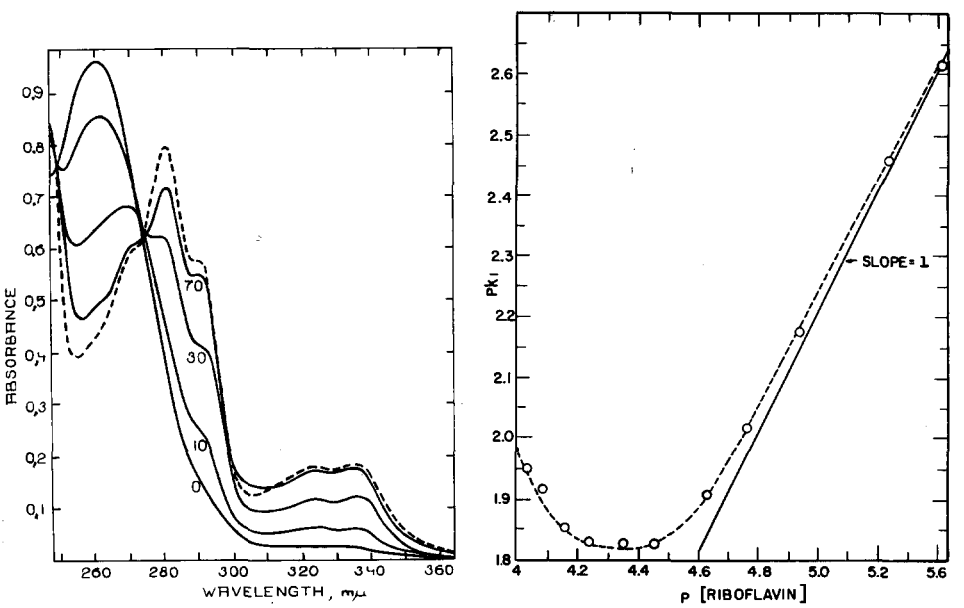


Fig. 1. Spectra of a mixture of riboflavin (0.05 mM) and ethyl 1,2-dihydro-2-naphthoate (0.125 mM) observed *versus* a 0.05 mM riboflavin blank after successive times of irradiation with white light. Solid curves are for 0, 10, 30, and 70 min of irradiation. The dotted line is the spectrum of pure ethyl naphthoate. All solutions are cacodylate buffer (pH 6.08, 0.03 ionic strength). Irradiation was with a 15-W fluorescent tube at a distance of 30 cm.

Fig. 2. Negative logarithm of apparent first order rate constant (pk_1) for oxidation of ethyl 1,2-dihydro-2-naphthoate plotted against the negative logarithm of the riboflavin concentration (p[riboflavin]). Cacodylate buffer, pH 6.08, 0.03 ionic strength. Irradiation with a 15-W fluorescent lamp at a 30-cm distance.

Reaction kinetics

When the logarithm of the concentration of unoxidized ethyl dihydronaphthoate present, as estimated from the changes in absorbance at 330 mμ (or at 260 mμ), was plotted against time, a straight line was obtained. This apparent first order relationship was observed for the photochemical oxidation as well as for that in the dark. A pseudo first order relationship will be expected for such a photochemical reaction only if the riboflavin concentration is very low or if there is complete mixing at all times in the cell. Since samples were withdrawn at frequent intervals with concomitant mixing, the latter condition is probably at least approximately satisfied. In any event, linear plots were always obtained. We have therefore expressed the results in terms of apparent first order rate constants.

The dependence of the reaction rate upon the riboflavin concentration is shown in Fig. 2 where the negative logarithms of the apparent rate constants are plotted against the negative logarithms of the riboflavin concentrations. At low flavin concentrations, the rate is nearly proportional to the riboflavin concentration. (Strict proportionality would require a slope of 1 as depicted by the heavy line drawn as an asymptote in Fig. 2.) This observation suggests that one molecule of riboflavin reacts with one molecule of ethyl dihydronaphthoate in the rate-limiting step. For purposes of comparison with the reaction in the dark, we have calculated from the apparent first order rate constants at low riboflavin concentrations an apparent second order rate constant. For a sample contained in a 1-cm silica cuvette 30 cm from the light, this constant is $5.4 \cdot 10^2$ l·mole^{-1}·min^{-1}.

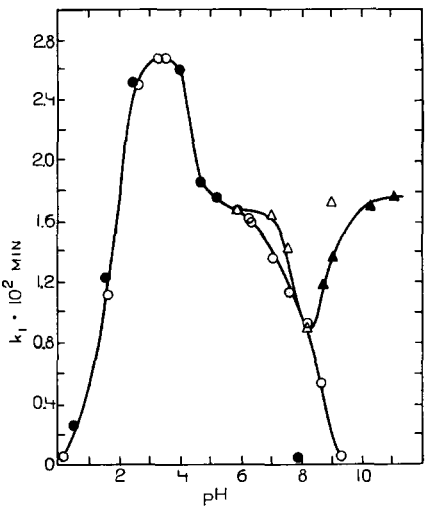
Dependence of photochemical reaction on pH

The rate of reaction shows an odd dependence on pH which is portrayed in Fig. 3. The rapid decrease in rate at the two extremes of pH is doubtless related to the loss of fluorescence of riboflavin under these conditions as noted by KAVANAGH AND GOODWIN[29]. Ionization of riboflavin in the unexcited state is governed by pK values of approx. 0.1 and 10.0 (ref. 3); however, the quenching of fluorescence will be controlled largely by the pK values for the photo-excited molecule together with appropriate rate constants. Also, the amount of quenching of fluorescence has been shown experimentally to depend on the concentration of various buffer ions including phosphate[30] and borate[31].

To test the hypothesis that the rate decrease at high pH results from dissociation of proton in the 3-position of the flavin, we studied the pH dependence of the oxidation reaction with 3-methyllumiflavin. This is shown by triangles in Fig. 3. We also measured rates with a number of other flavins including lumiflavin (Table I). The oxidation with the latter was only one-third as fast at pH 9.1 (borate buffer) as at pH 6.1 (phosphate buffer) whereas the rate of oxidation by 3-methyllumiflavin is decreased by only 20 % (Table I), and at higher pH values is just as rapid as at pH 6.1 (Fig. 3). A sharp decrease in rate between pH 7.5 and 9.5 in phosphate and borate buffers complicates the picture for 3-methyllumiflavin. However, it is clear that this compound, from which no dissociation of a proton from the 3-position can occur, reacts rapidly at high pH values at which no reaction takes place with riboflavin. The compound also continues to fluoresce brilliantly in this pH range. In a triethanolamine buffer at pH 7.88 (Fig. 3) the reaction rate was extremely slow, probably

because the photochemical oxidation of the buffer competed with the oxidation of dihydronaphthoate.

The dependence of the reaction with riboflavin upon pH in the intermediate pH range is also complex. The increase in rate below pH 5 is reminiscent of the rapid increase in rate with decreasing pH observed in the (non-photochemical) oxidation of *N*-propyldihydronicotinamide by riboflavin[3] which has been attributed to the



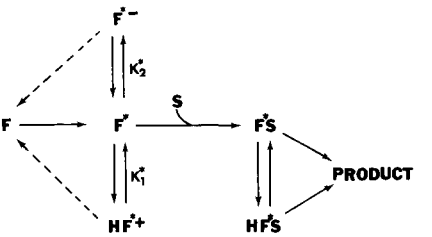


Fig. 3. Apparent first order rate constants for photooxidation of ethyl 1,2-dihydro-2-naphthoate *versus* pH. Buffers were of ionic strength 0.03 except at pH < 1. ○, ●, riboflavin; △, ▲, 3-methyllumiflavin. Buffers: ○, below pH 3, HCl; pH 3–4, formate; above pH 5, phosphate. ●, below pH 3, nitric acid; pH 3–6, acetate; pH 7.9, triethanolamine. △, phosphate except at pH 9.0 which was unbuffered. ▲, borate except at pH 11.0 which was carbonate. Irradiation by a 15-W fluorescent tube at a distance of 30 cm.

Fig. 4. Scheme for the photooxidation of dihydronaphthoate. F, F* are flavin and excited flavin, respectively. S is dihydronaphthoate.

TABLE I

RELATIVE RATES OF PHOTOOXIDATION OF ETHYL 1,2-DIHYDRO-2-NAPHTHOATE BY VARIOUS FLAVINS

Flavin	*pH 6.06**	*pH 9.14***
Riboflavin	1.00***	0
FMN	1.2	
FAD	0.2	
Isoriboflavin	0.5	0
Lumiflavin	0.9	0.3
3-Methyllumiflavin	1.0	0.8
2-Lumiflavimine	1.1	
Hydroxyethyl flavin (6,7-dimethyl-9-(2′-hydroxyethyl)isoalloxazine)	0.9	
Formylmethyl flavin (6,7-dimethyl-9-formylmethylisoalloxazine)	0.5	

* Phosphate buffer, 0.03 ionic strength.
** Borate buffer, 0.03 ionic strength.
*** Riboflavin at pH 6.06 is assigned a value of 1.00.

higher reactivity of protonated riboflavin. In the present case, it is possible that a complex between excited flavin and dihydronaphthoate reacts more rapidly if protonated than if not. The scheme given in Fig. 4 seems to provide the minimum number of reaction steps required to describe the behavior of the system. The state of the excitation is not implied by this scheme. Current evidence[11,12] suggests that the reactive form of flavin in the oxidation of amino compounds is the triplet state. The influence of ions (see below), particularly I^-, could indicate the involvement of the triplet state. Also, the mechanism of electron transfer has not been determined. Studies with amino compounds[8,11,12] or the ribityl side-chain[18–21] suggest radical mechanisms which would be consistent with our observations.

K_1^* and K_2^* represent the acid dissociation constants of the photoexcited flavin and the dotted arrows reactions by which the flavin may be returned to the ground state. The existence of the complex F*S and its protonated (and more reactive) form HF*S between excited flavin and dihydronaphthoate ester accounts for the variation of rate with pH in the middle range. The sharp drop in the reaction rate below pH 3 suggests that in this region the reaction of F* to F*S becomes rate-limiting as the flavin is deactivated through rapid loss of energy from HF*.

Reaction with other isoalloxazine derivatives

Relative rates of a number of flavins are given in Table I. The rate of reaction of flavin adenine dinucleotide is only one-fifth that of riboflavin. This decreased rate probably results in large part from the internal complexation of the flavin with the adenine moiety which is known to decrease greatly the fluorescence of the flavin[32]. Steric hindrance by the adenine group may also be a factor. Isoriboflavin (methyl groups in Positions 5 and 6 rather than 6 and 7 of the ring system) possesses a weaker fluorescence than riboflavin and also reacts more slowly.

Effect of solvent

The rate of oxidation diminished quite rapidly upon addition of increasing concentrations of ethanol and at 62 % ethanol by volume, the rate had decreased to an unmeasurably low value. Several explanations might be proposed, the seemingly best being that the ethanol becomes a competing substrate for the photoexcited flavin[17]. The rate was only weakly dependent upon the ionic strength of the solution. Increasing the ionic strength from near zero to 0.6 led to a decrease in rate of about 25 %. Addition of various inorganic salts (0.1 mM salt in pH 6.08 cacodylate buffer) either had no effect on the rate or caused a decrease in rate. Salts of Fe^{3+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Br^- and Cl^- had no effect but those of Fe^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Ag^+, $S_2O_3^{2-}$, SO_3^{2-} and I^- caused about a 30 % inhibition. The inhibition appears to reflect principally a quenching of the flavin fluorescence and/or triplet state.

Effect of temperature

An unusual result was obtained when the photooxidation was carried out at various temperatures. The rate decreased with increasing temperature; the slope of the Arrhenius plot (Fig. 4) giving a heat of activation of -2.25 kcal/mole. This negative heat of activation suggests the intermediate formation of a reactive complex between the photoexcited flavin and the dihydronaphthoate ester such as we have proposed to explain the pH dependence of the reaction.

The reaction in the dark

The same reaction occurs much more slowly in the dark at elevated temperatures. This was shown by heating the reaction mixture at 95° in a cacodylate buffer of pH 6.08 (measured at room temperature) of ionic strength 0.03. The same characteristic change in absorption spectrum occurred as that shown in Fig. 1 for the photoreaction with ethyl 2-naphthoate being the sole product. The reaction is much slower at 95° in the dark than at 25° in the light, the second order rate constant at pH 6.08 being 25 $l \cdot mole^{-1} \cdot min^{-1}$. The time required for half reaction was about 9 h in the dark at 95° but only 24 min in the light at 25°. The heat of activation of the reaction in the dark is about +14.6 kcal/mole as calculated from the plot in Fig. 5.

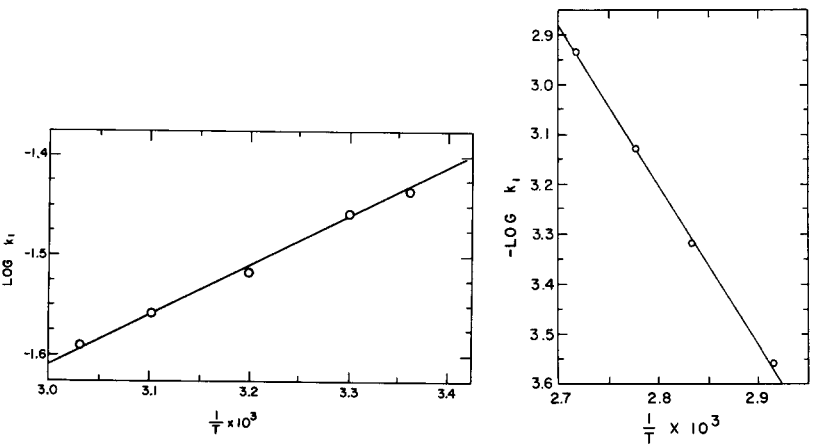


Fig. 5. Arrhenius plot for photooxidation of ethyl 1,2-dihydro-2-naphthoate by riboflavin. Logarithm of apparent first order rate constant *versus* the reciprocal of absolute temperature. Riboflavin concentration, 47 μM; cacodylate buffer, pH 6.08; 0.03 ionic strength.

Fig. 6. Arrhenius plot for thermal oxidation of ethyl 1,2-dihydro-2-naphthoate by riboflavin. Logarithm of apparent first order rate constant against the reciprocal or absolute temperature. Riboflavin concentration, 47 μM; cacodylate buffer, pH 6.08; 0.03 ionic strength.

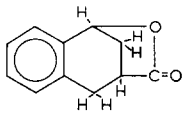
Oxidation of other dihydro-2-naphthoates

The ethyl esters of the isomeric 3,4- and 1,4-dihydro acids do not undergo photooxidation under the conditions described above. No change was observed in the spectra of the reaction mixtures during irradiation. These results may be compared with the thermal oxidation of the dihydronaphthalenes[33,34] by benzoquinone and by *o*- and *p*-naphthoquinones. In this latter case, 1,4-dihydronaphthalene was oxidized about 15 times as rapidly as 1,2-dihydronaphthalene. The presence of the carboxyl group and its activating influence may account for the difference in the two systems.

The free dihydro-2-naphthoic acids were also tested for ability to undergo photooxidation. While some oxidation probably occurred, the 1,2-dihydro acid underwent a competing reaction as revealed by spectral analysis. We suggest the formation of the internal lactone, II, as a possible concurrent reaction. This reaction is analogous to the reaction of the acid with iodine to yield the iodolactone[26].

The 3,4-dihydro acid was not oxidized at pH 3 and 6, but was converted to a compound with an unidentifiable spectrum similar to that of an alkyl-substituted benzene. Changes in the spectrum during the photoreaction of the 1,4-dihydro acid

suggested a very small conversion into 2-naphthoic acid at pH 3 but not at pH 6. However, because the ratio of the absorbance at 290 and 330 mμ in the product did not correspond to that of 2-naphthoic acid no further studies were made.



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