The hydrolysate of DNP-(A) yielded mono-DNP-cystine (R_f = 0.62 in phenol-citrate-cyanide-ammonia mixture), and in addition two yellow spots, one ninhydrin-reactive with R_f = 0.85, and the other ninhydrin-negative with R_f = 0.93. That the latter two spots arise by decomposition of mono-DNP-cystine during the hydrolytic procedure was shown by subjecting this derivative to the boiling acid solvent and taking paper chromatograms at hourly intervals. The two spots gradually made their appearance with increasing intensity as the boiling proceeded. A similar experiment with DNP-(A) revealed the same phenomenon. No trace of cystine (R_f = 0.22) or of di-DNP-cystine (R_f = 0.81) was ever detected among the hydrolytic products under these conditions. However, in a refluxing solvent composed of 3 *N* HCl in 25% formic acid, DNP-(A) yielded, in addition to mono-DNP-cystine, two spots on paper; one of these was yellow and ninhydrin-insensitive (R_f = 0.76), and the other was colorless, ninhydrin-reactive, and identified unmistakably as due to cystine.

The hydrolysate of DNP-(A + B) yielded clear evidence of cystine in the aqueous extract (R_f = 0.22) and di-DNP-cystine (R_f = 0.81) in the ether extract. In addition there was clear evidence for the presence of mono-DNP-cystine as well as for small amounts of its decomposition products. In all cases mono-DNP-cystine was recognized by its solubility in aqueous media and by its chromatographic behavior, moving as a yellow spot which on development with ninhydrin became purple-brown in color.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN CO.]

Metabolite Inhibitors. I. 6,7-Dimethyl-9-formylmethylisoalloxazine, 6,7-Dimethyl-9-(2′-hydroxyethyl)-isoalloxazine and Derivatives[1]

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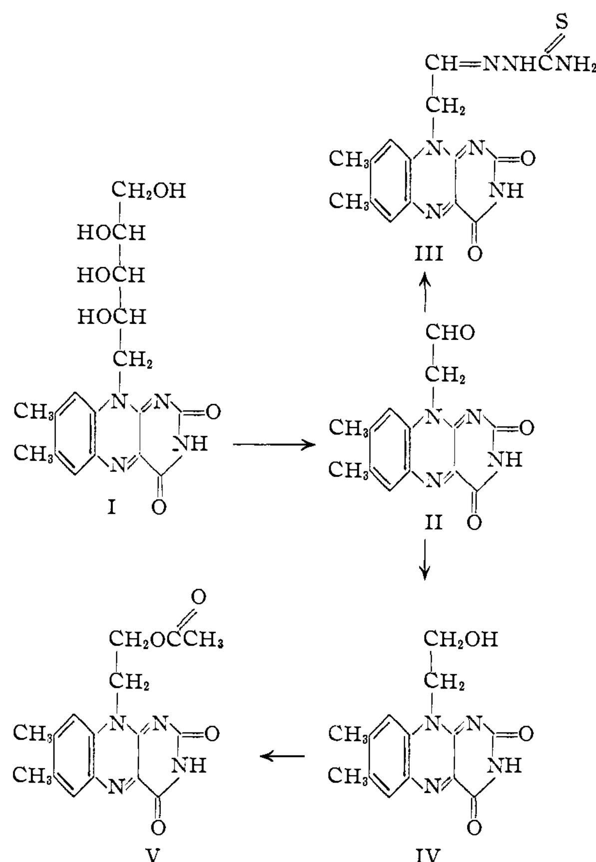
A series of biologically active riboflavin antagonists—6,7-dimethyl-9-formylmethylisoalloxazine (II), 6,7-dimethyl-9-(2'-hydroxyethyl)-isoalloxazine (IV) and 6,7-dimethyl-9-(2'-acetoxyethyl)-isoalloxazine (V)—has been prepared. Other derivatives of II and IV also have been made. Physical properties are presented. The use of ultraviolet absorption spectra on the quantitative level and saponification numbers is demonstrated.

Introduction

The development of a potent riboflavin antagonist is of interest for fundamental studies in metabolism as well as for investigation of possible chemotherapeutic activity. That analogs of riboflavin inhibit flavoenzymes or influence riboflavin metabolism in a variety of biological systems provided a reason to prepare a number of isoalloxazines for evaluation of their therapeutic activity against experimental cancer and as antiparasitic and antimicrobial agents. There are only a few riboflavin antagonists of the isoalloxazine structure effective in either animal or both animal and microorganism systems.[2-5] The structural criteria for biological activity appear to be vicinal alkyl groups in the 5,6- or 6,7-positions and sugar residues of pentose or hexose origin in the 9-position. This paper constitutes the first in a series which will discuss the chemistry and biochemistry of a group of isoalloxazine compounds with a 2-carbon residue in position 9 having interesting biological activity, possible therapeutic value and reversible riboflavin antagonism in animals as well as in systems of microörganisms. Specifically, this report covers 6,7-dimethyl-9-formylmethylisoalloxazine (II), 6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine (IV) and certain of their derivatives.

During the period in which the structure of riboflavin was being elucidated, Kuhn[6] reported that the oxidation of riboflavin with an excess of lead tetraacetate in glacial acetic acid gave 78% of the



theoretical amount of formaldehyde expected from the vitamin B_2 compound if it had only one primary aliphatic alcohol group in the tetrahydroxyamyl sidechain. However, no other identifiable compounds were isolated from this reaction which was carried out strictly for analytical purposes.

(1) Presented at the 126th Meeting of the American Chemical Society, New York, N. Y., September, 1954.

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(3) H. v. Euler and P. Karrer, *Helv. Chim. Acta*, **29**, 353 (1946).

(4) G. A. Emerson, E. Wurtz and O. H. Johnson, *J. Biol. Chem.*, **160**, 165 (1945).

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Later, Petering and Weisblat[7] prepared a pteridyl aldehyde by a similar oxidation of a polyhydroxyalkyl sidechain in the pterine series. The degradation of riboflavin (I) to the aldehyde II has been successfully accomplished in this Laboratory by the oxidation of the vitamin with glycol cleavage reagents. Carbonyl derivatives of the aldehyde can be obtained directly from such oxidizing mixtures by reaction with the appropriate reagent. The yields of the derivatives were, to all intents and purposes, quantitative indicating that there was no decomposition of the aldehyde in solution under the experimental conditions.

Since the known antagonists of the isoalloxazine category are polyalcohols, it was of interest to determine the biological activity, if any, of the primary alcohol derived from II. The reduction of II was undertaken and successfully accomplished with sodium borohydride. Many of the polyhydroxyalkylisoalloxazines including riboflavin have been acetylated to their respective tetra- and pentaacetates.[8-13] The acetate of IV, V, was made by the use of an acetylating mixture of acetic anhydride and pyridine. Although only riboflavin tetraacetate had been tested for microbiological activity and found to be inactive,[14] it was considered worthwhile to note whether such a modification in the functional group of the sidechain would alter the activity of IV to any degree.

Experimental

6,7-Dimethyl-9-formylmethylisoalloxazine Hydrate (II).—To a stirred suspension of 5.7 g. (0.015 mole) of riboflavin in 150 ml. of 2 *N* sulfuric acid cooled in an ice-bath and protected from light was added 90 ml. of an aqueous solution of 12.6 g. (0.055 mole) of periodic acid (H_5IO_6). The mixture was stirred at ice-bath temperature for 30 minutes and then allowed to warm to room temperature until all solids went into solution (about one hour). The *p*H was adjusted to 1.5 by the addition of solid sodium carbonate. A few drops of capryl alcohol prevented excessive foaming. At this point a small amount of precipitate formed. Darco, 1.5 g., was added to the mixture, and after 30 minutes of stirring it was filtered. The *p*H of the filtrate was carefully adjusted to 3.8 with more solid sodium carbonate. II precipitated and was removed by filtration. The solid was washed thoroughly with cold water until the filtrate contained no demonstrable sulfate ion (aliquots tested with lead acetate solution). It was then washed with alcohol and ether and dried *in vacuo*. The yield of II was 3.4 g., 75.1%, m.p. 270.5–271.0°[15] dec.

Anal. Calcd. for $C_{14}H_{12}N_4O_3 \cdot H_2O$: C, 55.6; H, 4.64; N, 18.5. Found: C, 56.1; H, 4.38; N, 18.1.

II Thiosemicarbazone (III).—II hydrate, 3.02 g. (0.010 mole), was dissolved in 300 ml. of 50% glacial acetic acid by warming on steam-bath with precautions taken to protect dissolved compound from light. To this solution was added a solution of 1.82 g. (0.020 mole) of thiosemicarbazide in 80 ml. of hot water. An orange precipitate formed immediately. To complete the reaction, the mixture was stirred for an hour. The precipitate was filtered, washed with hot water, alcohol and ether, and then dried *in vacuo*. The yield of the compound was 3.20 g., 96.4%, m.p. 236.5–237.0°[15] dec.

Anal. Calcd. for $C_{15}H_{15}N_7O_2S$: C, 50.4; H, 4.20; N, 27.5; S, 8.96. Found: C, 50.6; H, 4.52; N, 26.4; S, 8.97.

II Oxime.—A solution of II was prepared as above and protected from light. Sodium acetate, 2.00 g., was added and then a solution of 1.38 g. (0.020 mole) of hydroxylamine hydrochloride in 50 ml. of water. As the precipitation of the product was very gradual, the reaction mixture was warmed on a steam-bath whereupon a yellow solid came down rapidly. The mixture was cooled to room temperature and then centrifuged. The extreme fineness of the crystalline product made paper filtration difficult. The compound was washed with water, alcohol and ether and dried *in vacuo*. The yield was 2.40 g., 80.2%, m.p. 260.0–261.0°[15] dec.

Anal. Calcd. for $C_{14}H_{13}N_5O_3$: C, 56.2; H, 4.35; N, 23.4. Found: C, 56.0; H, 4.35; N, 22.9.

III from Oxidation of Riboflavin with Lead Tetraacetate.—It is possible to prepare carbonyl derivatives of II, such as the thiosemicarbazone, directly from the reaction mixture. Where lead tetraacetate is used for this purpose, obviously lead must be removed if one is to investigate the compounds for biological activity. However, isolations for identification purposes only do not require this step. Throughout these separations, though, it must be kept in mind that the dissolved aldehyde is a very sensitive compound and subject to photolytic decomposition.

A solution of 8.0 g. of lead tetraacetate in 80 ml. of glacial acetic acid was prepared by adding the solid to the acid preheated to 50° and then warming to 65°. This was added with vigorous stirring and in several portions to a cold solution of 2.0 g. of riboflavin in 80 ml. of 0.1 *N* sodium hydroxide. The reaction mixture was maintained in an ice-bath throughout the preparation. Several minutes after the addition of the final portion of lead tetraacetate, a solution of 2.0 g. of thiosemicarbazide in 10 ml. of hot water was poured into the mixture. The resultant solution was allowed to warm slowly to 45° whereupon a reddish-orange solid precipitated. After the mixture was cooled to room temperature, it was filtered. The residue was washed thoroughly with 5% aqueous acetic acid; then it was washed with water, alcohol and ether, and dried *in vacuo*. The product weighed 16.2 g., 85.2% yield. Spectrographic analysis showed the presence of not more than 0.43% lead. Infrared data confirmed the structure of the compound.

Anal. Calcd. for $C_{15}H_{15}N_7O_2S$: S, 8.96. Found: S, 9.28.

6,7-Dimethyl-9-(2'-hydroxyethyl)-isoalloxazine (IV).—30.2 g. of 6,7-dimethyl-9-formylmethylisoalloxazine hydrate (II) was dissolved in 1.0 l. of cold 0.1 *N* sodium hydroxide. This solution and all subsequent reaction mixtures were protected from light. To the alkaline solution of II was added with vigorous stirring 3.7 g. of sodium borohydride in 25 ml. of water. The mixture was allowed to react with stirring at room temperature for two hours after which it was cooled in an ice-bath and the *p*H adjusted to 4.0–4.5 with glacial acetic acid. The precipitate which formed was removed by filtration and washed thoroughly with water, dilute acetic acid and acetone. The product, dried *in vacuo* at 75°, weighed 26.0 g., 91.0% yield, m.p. 300.5–301.0°[15] dec.

Anal. Calcd. for $C_{14}H_{14}N_4O_3$: C, 58.7; H, 4.9; N, 19.6. Found: C, 58.7; H, 4.7; N, 19.6.

6,7-Dimethyl-9-(2'-acetoxyethyl)-isoalloxazine (V).—25.0 g. of IV, 900 ml. of anhydrous pyridine and 46 ml. of acetic anhydride were heated together at reflux temperature for 30 minutes. The reaction mixture was filtered while hot through a Büchner funnel. The filtrate was refrigerated at 5° for at least 48 hours. The crystals were collected by filtration, washed with ether and vacuum dried overnight at 75°. A second crop was obtained by the concentration of the total filtrate to one-third its original volume. The two crops, 27.1 g., were combined and recrystallized from boiling glacial acetic acid. The crystalline product weighed 21.8 g., 75.9% yield, m.p. 230.0–232.0°[15] dec.

Anal. Calcd. for $C_{16}H_{16}N_4O_4$: C, 58.6; H, 4.9; N, 17.1; CH_3CO, 13.1. Found: C, 59.1; H, 4.9; N, 17.9; CH_3CO, 12.9.

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(14) E. E. Snell and F. M. Strong, *Enzymologia*, **6**, 186 (1949).

(15) All melting points are uncorrected.

Esters of IV.—In addition to the acetate, several other organic esters of IV were prepared. Among these were the propionate, butyrate, acid succinate, benzoate, cyclopentanopropionate, laurate and palmitate. Where the acid anhydride was available, it was used with pyridine as described above for the acetate. Otherwise the acid chloride was used.

Saponification Equivalents.—The study of the saponification equivalents of this series of hydroxyalkylisoalloxazine esters, in particular the acetate, required certain modifications to be made in standard procedures.[16,17] Standard solutions of aqueous 0.1 *N* sodium hydroxide and 0.1 *N* hydrochloric acid were used. Titration readings were obtained from a Beckman *p*H meter, model G, with outside electrodes.

TABLE I

MOLAR EXTINCTION COEFFICIENTS

Compound	Maxima mμ	Maxima $\epsilon \times 10^{-3}$	Minima mμ	Minima $\epsilon \times 10^{-3}$
I	270	35.4	240	10.5
	355	11.6	295	1.77
			395	5.45
II	267	36.4	238–239	11.2
	350–352	10.2	296–300	2.38
			392–394	5.89
III	270	43.8	240	17.9
	353–355	11.2	316–318	6.64
			392–396	6.32
II oxime	269–270	35.7	242	12.0
	353–357	12.0	294–296	2.36
			392–396	6.36
IV	270	36.0	240	9.69
	355	11.8	295	2.00
			395	5.76
V	270	36.5	240	9.80
	355	12.1	295	2.09
			395	5.86

The procedure which consistently gave accurate results was as follows. A known weight of the compound to be analyzed, usually 400 mg., was dissolved in a measured volume of 0.1 *N* sodium hydroxide which was an excess over the amount sufficient for hydrolysis. This solution was heated under reflux for an hour. After cooling it was made up to approximately 200 ml. with distilled water. The solution was then titrated electrometrically with standard acid. Within the range where neutralization occurred readings were taken every 0.2 ml. A plot of *p*H against ml. of 0.1 *N* acid was made and the end-point of the titration determined graphically.

Ultraviolet Absorption Spectra.—All ultraviolet absorption spectra were determined with a Beckman quartz spectrophotometer, model DU. The compounds were dissolved in 0.1 *N* aqueous sodium hydroxide, the solutions protected from light throughout their preparations, and the data obtained as rapidly as possible. With the exception of riboflavin (10 mg./l.), the concentration of compounds used was 8 mg./l. The silica absorption cells had a 1-cm. light path. Measurements from 230 to 400 mμ were taken at room temperature. The values for the maxima and minima are given in Table I.

Infrared Spectra.—The pertinent infrared data are summarized in Table II.

Fluorescence.—IV and V are very similar to riboflavin in their fluorescent characteristics.[18] When their aqueous solutions are exposed to ultraviolet light both fluoresce greenish yellow. The aqueous solutions are also more stable to storage than that of riboflavin and can, therefore, be used as standards for the quantitative fluorescence measurements important in biochemical studies. Solutions of IV and V can be reduced by sodium hydrosulfite with the concomitant quenching of the fluorescence. This returns on shaking the solutions with air. Alkali completely destroys the fluorescence.

Paper Chromatography.—I, IV and V were studied by circular paper chromatographic methods using on Whatman No. 4 paper the two solvent systems: 1, *n*-butyl alcohol saturated with water, and 2, *n*-butyl alcohol, water and acetic acid (7:2:1). The discs were examined under ultraviolet light to determine the bands, and the R_f values were found to be

	System 1	System 2
I	0.30	0.53
IV	.50	.66
V	.70	.77

Biological Activity.[19]—Both II and III were found to be reversible riboflavin antagonists in the rat and to have other biological activity. In this Laboratory, IV and V have been shown to be potent competitive antagonists of riboflavin in growing rats and against *L. casei*. Substantial antifungal, antibacterial and antiparasitic activities have been established for the latter two compounds. In addition, they show antitumor activity against the Murphy-Sturm lymphosarcoma in rats.

TABLE II

INFRARED DATA

Functional group	Band, cm.⁻¹ I	II	III	IV	V
OH and NH	3325	3404, 3250	3535, 3467 3325, 3280	3220	3276, 3206
Flavin C=O	1714, 1672	1708, 1655	1706, 1681	1707, 1670 1576	1718, 1654
C=C	1579	1578, 1544 (C=C, C=N)	1575	1502	1682
C=N	1549		1542	1544	1544
Alcohol C—O	1156				
Acetate C=O					1740
Acetate C—O					1247
C=S			1612		

Discussion

Saponification Number.—It was known that the alkaline hydrolysis of both riboflavin and lumiflavin resulted in the formation of urea and a quinoxalinecarboxylic acid.[20–23] On the basis of the close similarities in structure among the compounds, it was anticipated that IV and V would likewise hydrolyze under alkaline conditions to urea

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(23) R. Kuhn and F. Weygand, *ibid.*, **67**, 1409 (1934).

and a quinoxalinecarboxylic acid. The application of saponification number analysis to this series of esters required that the alkaline degradation be quantitative. The saponification numbers experimentally determined—I (commercial grade), mol. wt. 376, 354; IV, mol. wt. 286, 286; and V, mol. wt. 328, 160–163—indicated without question that the alkaline degradation was quantitative. Although this work is admittedly exploratory, the method does show promise as an excellent analytical tool for isoalloxazines. However, it was not the purpose of this paper to define the scope of such analytical procedures and the method has not been investigated in detail.

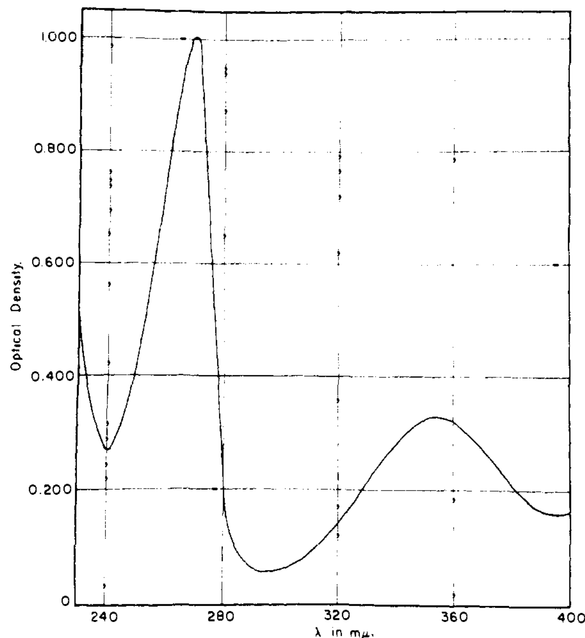

Fig. 1.—6,7-Dimethyl-9-(2'-hydroxyethyl)-isoalloxazine in 0.1 N NaOH, 8 mg./l.

Ultraviolet Absorption Spectra.—In 0.1 N sodium hydroxide solution, riboflavin, IV and V have ultraviolet absorption curves based upon molar extinction coefficients which are coincidental in virtually every respect. The obvious conclusion is that the same electronic species must be present in each case. This suggested that the nature of the 9-sidechain had no effect upon the electronic configuration of the 6,7-dimethylisoalloxazine nucleus so long as it was of hydroxyalkyl or polyhydroxyalkyl derivation. On this assumption then, it was possible to reduce the defining equation for the molar extinction coefficient[24] to a simple inverse

$$\epsilon_\lambda = \frac{1}{md} \log \frac{I_{0\lambda}}{I_\lambda}$$

proportionality between the optical density (O.D.) as read directly on the Beckman DU and the molecular weight of the compound. The derivation is

$$\epsilon_\lambda = \frac{1}{md} \text{ O.D.}$$

$$= \frac{1}{(\text{concn./mol. wt.})\, d} \text{ O.D.}$$

Since ϵ_λ and d are constants, then

$$\text{O.D.} = k \text{ (concentration/mol. wt.)}$$

The standard used in this Laboratory was IV having an O.D. of 1.010 at 270 mμ for a 0.1 N sodium hydroxide solution containing 8 mg./l (Fig. 1). Within the limits of Beer's law, this should permit calculation of the absorption at the 270 mμ maximum of any 6,7-dimethylisoalloxazine of this series. Studies not only on I and V but also on other esters of IV substantiated this procedure as a quick and accurate method for the analysis of the isoalloxazines herein considered.

There is not much information in the literature concerning the ultraviolet absorption spectra of isoalloxazines in general.[25–29] What is available does indicate that most absorption curves resemble that of riboflavin. Adams, Weisel and Mosher[27] published absorption curves in 0.1 N sodium hydroxide for riboflavin and 6,7-dimethyl-9-(γ-diethylamino-β-hydroxypropyl)-isoalloxazine monohydrochloride dihydrate. Data obtained from their graphs are in conformity with like values listed in this paper (Table I).

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