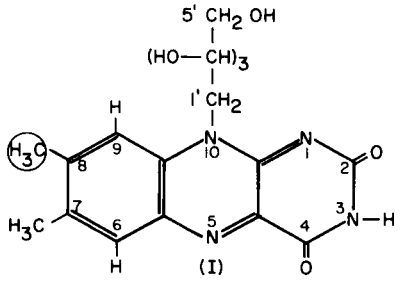
Flavin Derivatives *via* Bromination of the 8-Methyl Substituent (1)

Donald B. McCormick

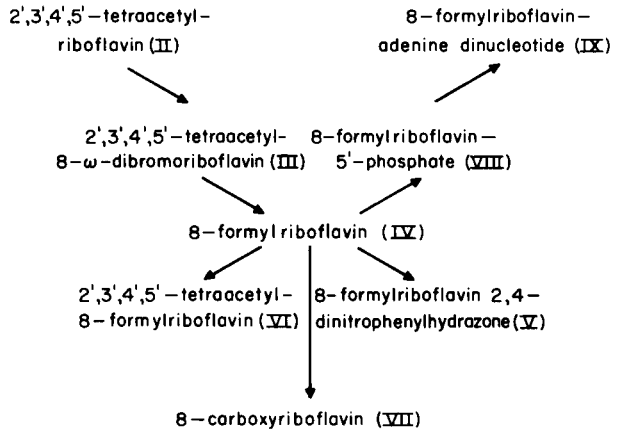
Graduate School of Nutrition and Section of Biochemistry and Molecular Biology
Cornell University

Several types of flavins have been synthesized in which both 7- and 8-methyl substituents of the natural vitamin, riboflavin, have been replaced in starting compounds with chloro (2) or bromo (3) groups and in which either one or the other methyl is replaced by such halogen functions (4,5). Direct halogenation of the 7,8-dimethylflavin nucleus in acid media leads to substitution at position 9 in the benzenoid portion (6). Also, a halogenated derivative of the principal flavin coenzyme, flavin-adenine dinucleotide, has been made by 8-bromination of the adenylate portion prior to incorporation into the final dinucleotide (7).

Until now, no satisfactory means has been made available for direct halogenation at either methyl group in position 7 or 8 of a flavin. Though halogenation can be effected by photochemical means in the methyl groups of many such aromatic compounds, the presence of the photolyzable 10-polyol side chain in flavins eliminates this method for directly obtaining the intact ω-haloflavin. However, the known reactivity of the 8-methyl group of flavins, especially in the formation of biflavins under alkaline anhydrous conditions (8), suggested the probability for direct bromination at this position (circled) in a suitably side-chain protected form of riboflavin (I).



The present paper describes the dibromination of tetraacetylriboflavin at the 8-methyl and hydrolysis of this compound to 8-formylriboflavin (9) which can be reacted with conventional carbonyl reagents, reacetylated, converted to coenzyme analogs, or oxidized to the carboxyl derivative as shown in the following scheme. Such chemically reactive 8-substituted flavins offer a means for syntheses of further analogs that may be of considerable aid in delineating the biological function of the 8-methyl



group of flavins through which covalent attachment to such specific proteins as succinic dehydrogenase appears to occur (10).

Riboflavin (I) was exhaustively acetylated with acetic anhydride and a trace of perchloric acid to form the tetraacetyl derivative (II) which, in turn, was reacted with excess bromine and pyridine in dioxane to form a complex of the brominated tetraacetylriboflavin with dibromopyridine. The complex was disrupted in aqueous acid to yield the tetraacetyl-8-ω-dibromoriboflavin (III) which was subsequently hydrolyzed under reflux to 8-formylriboflavin (IV) in good yield. The formylflavin could be condensed with 2,4-dinitrophenylhydrazine under usual conditions to yield the hydrazone (V), peracetylated to tetraacetyl-8-formylriboflavin (VI), or readily oxidized to 8-carboxyriboflavin (VII). Also, the formylflavin was phosphorylated at the 5'-hydroxymethyl function to form an analog (VIII) of flavin mononucleotide (FMN) which could be condensed with adenosine 5'-phosphoromorpholidate to yield the analog (IX) of flavin-adenine dinucleotide (FAD) purified by anion-exchange column chromatography. Elemental analyses determined for all the fore-mentioned derivatives of riboflavin are given in Table I.

That dibromination of the 8-methyl of the flavin occurred is proven by comparisons of the NMR spectra of the starting and product tetraacetyl derivatives as summarized by the data in Table II. Whereas tetraacetylriboflavin in deuterated chloroform exhibits peaks at δ values of

TABLE I

Elemental Analyses of Flavin Derivatives

	Compound	Calculated			Found		
		C	H	N	C	H	N
II	$C_{25}H_{28}N_4O_{10}$	55.14	5.18	10.29	55.16	5.26	10.45
III (a)	$C_{25}H_{26}N_4O_{10}Br_2$	42.75	3.73	7.99	42.44	3.80	7.80
IV	$C_{17}H_{18}N_4O_7$	52.30	4.65	14.36	52.58	4.46	14.18
V	$C_{23}H_{22}N_8O_{10}$	47.10	3.76	19.11	47.02	3.83	19.07
VI	$C_{25}H_{26}N_4O_{11}$	53.76	4.69	10.03	53.58	4.69	10.08
VII	$C_{17}H_{18}N_4O_8$	50.25	4.46	13.79	50.63	4.33	13.50
VIII (b)	$C_{17}H_{18}N_4O_{10}LI\cdot 2H_2O$	40.65	4.41	11.15	40.93	4.51	11.38
IX	$C_{27}H_{29}N_9O_{16}Li_2\cdot 2H_2O$	38.16	4.15	14.84	38.26	4.26	14.60

(a) Bromine was calculated to be 22.75 and found 22.58. (b) Phosphorus was calculated for the monolithium dihydrate salt to be 6.17 and found 6.09. (c) Phosphorus was calculated for the dilithium dihydrate salt to be 7.29 and found 7.14.

TABLE II

Characteristics of NMR Spectra of Flavin Derivatives (a)

		Aryl-H		Aryl-CH	Aryl-CH_3	
Substance	Solvent	6	9		8	7
Tetraacetylriboflavin	$CDCl_3$	8.01	7.68	----	2.58	2.47
Tetraacetyldibromoriboflavin	$CDCl_3$	8.30	8.05	7.00	----	2.62
Riboflavin	CF_3CO_2D	8.30	8.20	----	2.82	2.69
Formylriboflavin	CF_3CO_2D	8.40	8.25	9.08	----	2.72

(a) Chemical shift is given in δ values (ppm) with tetramethylsilane as internal reference.

2.47 for the 7-methyl and 2.58 for 8-methyl substituent, only one three-proton peak is observed at 2.62 for the tetraacetyldibromoriboflavin, but a new one-proton peak appears at 7.00. Hence, one of the methyl functions has had two hydrogens displaced by bromine, and the remaining hydrogen reflects its more acidic character by the large down-field shift. As expected from the presence of the dibromomethyl substituent, the position of the *ortho* hydrogen at position 9 is somewhat down-field shifted to 8.05 from the original 7.68 and a lesser effect of 8.30 from 8.01 is seen with the *meta* hydrogen at position 6. These secondary effects on the aryl hydrogens are in agreement with the known shifts in position of benzenoid protons caused by a withdrawing substituent (11). The same reason accounts for the slight down-field shift of the single remaining 7-methyl from 2.47 to 2.62. Hydrolysis of tetraacetyldibromoriboflavin to remove the acetyl blocking groups and replace the bromo by hydroxyl groups leads to the aldehyde as also confirmed by the NMR characteristics given in Table II in comparison with riboflavin in deuterated trifluoroacetic acid. Again the absence of the 8-methyl group, down-field shifts of the aryl hydrogens and remaining 7-methyl (already somewhat shifted because of the acid solvent which has protonated the flavin), and appearance of a new peak at 9.08 compatible with the aldehydic proton (11) are observed. Overall, the assignments are given for the aryl hydrogens and methyls agree well with those reported earlier for the 7,8-dimethylisoalloxazine nucleus (6,12).

Absorption spectra of the 8-formylflavins, illustrated with the FAD analog in Figure 1, are rather characteristic. The absorption maximum near 450 mμ ($\epsilon \times 10^{-3} = 11.5$)

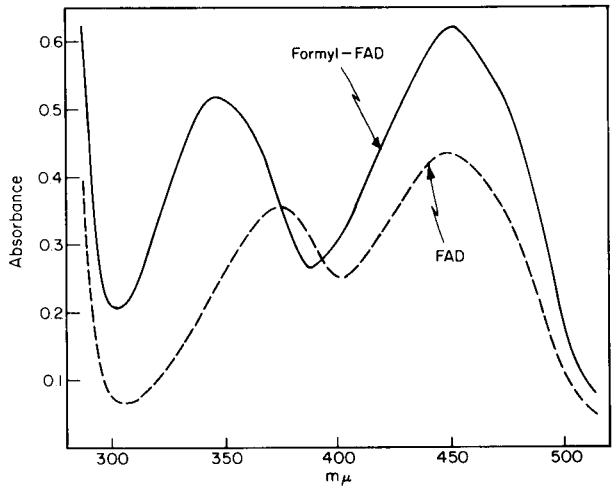


Figure 1. Absorption Spectra of 8-Formyl-FAD and FAD. Flavins were approximately 4 x 10^{-5} *M* in 0.1 *M* sodium phosphate buffer at *p*H 7.0.

for the neutral quinonoid species is similar to the parent flavin, but the near-ultraviolet maximum at 370 mμ of the latter is blue-shifted by about 25 mμ to a maximum near 345 mμ for the formyl analog.

EXPERIMENTAL

Microanalyses were by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. The NMR spectra were determined with 0.1 *M* flavins and an ambient probe temperature in a Varian A-60A spectrometer. The light absorption spectra were measured with a Cary Model 14 recording spectrophotometer.

2′,3′,4′,5′-Tetraacetylriboflavin (II).

This known compound (13) was synthesized by suspending 2.5 g. of riboflavin in 200 ml. of glacial acetic acid-acetic anhydride (1:1), effecting solution by dropwise addition of 0.5 ml. of 70% perchloric acid, stirring for 30 minutes at 40°, cooling in an ice bath, and diluting with an equal volume of cold water. The diluted solution was extracted twice with 200-ml. quantities of chloroform, the combined chloroform extracts washed twice with 100-ml. portions of water, and the organic phase dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure at 50° to yield 3.5 g. (95%) of the tetraacetylriboflavin with a melting point of 250-252°.

2′,3′,4′,5′-Tetraacetyl-8-ω-dibromoriboflavin (III).

Tetraacetylriboflavin (3.5 g.) was dissolved in 25 ml. of warm dioxane into which 2.5 ml. of pyridine followed by 2.5 ml. of bromine were stirred. The solution was refluxed for 2 hours and evaporated to a syrup. A small aliquot of this crude mixture stirred into acetone and diethyl ether gave a reddish-brown precipitate which by analysis contained 31.60% bromine and was in reasonable agreement for a 1:1 molecular complex of tetraacetyldibromoriboflavin with dibromopyridine which would contain 30.88% bromine. The entire mixture was then stirred into 250 ml. of chloroform, shaken briefly with an equal volume of 0.5 *N* hydrochloric acid, and the organic phase washed twice with 250-ml. portions of water. The chloroform solution was dried over anhydrous sodium sulfate, the volume reduced to about 10 ml. by evaporation under reduced pressure, and the concentrated solution stirred into 100 ml. of ether to precipitate crude product. The precipitate was collected by filtration, resuspended in 5 ml. of methanol and 45 ml. of ether, again collected by filtration, rinsed with ether, and dried to give 2.5 g. (50%) of the tetraacetyldibromoriboflavin.

8-Formylriboflavin (IV).

Tetraacetyldibromoriboflavin (2.5 g.) was dissolved in 50 ml. of warm 6 *N* hydrochloric acid and refluxed for one hour. The solution was evaporated to near dryness, stirred with 10 ml. of water and allowed to stand at room temperature overnight. The precipitate was collected by filtration, rinsed with methanol and ether, ground up in 25 ml. of warm chloroform, again collected by filtration and rinsed with methanol and ether, and dried to give 1,2 g. (90%) of the formylriboflavin.

8-Formylriboflavin 2,4-Dinitrophenylhydrazone (V).

A warm solution of 50 mg. of 2,4-dinitrophenylhydrazine in 0.2 ml. of concentrated hydrochloric acid and 1.5 ml. of ethanol was stirred into 25 ml. of hot 50% aqueous ethanol containing 50 mg. of the formylriboflavin. The solution was brought to boiling, cooled, and the precipitate collected by filtration. After drying, approximately 65 mg. (90%) of the hydrazone was recovered.

2′,3′,4′,5′-Tetraacetyl-9-formylriboflavin (VI).

Formylriboflavin (250 mg.) was stirred into 10 mg. of glacial acetic acid-acetic anhydride (1:1), 0.1 ml. of 70% perchloric acid was added, and the solution was warmed at 40° for 30 minutes. After cooling, the solution was diluted with 25 ml. of water and extracted with 25 ml. of chloroform. The chloroform extract was washed with equal volumes of water, 0.1% sodium bicarbonate, and again water. The organic phase was dried over anhydrous sodium sulfate, filtered, and the solvent removed under reduced pressure to give 300 mg. (90%) of the tetraacetylformylriboflavin.

8-Carboxyriboflavin (VII).

The formylriboflavin was readily oxidized to the carboxylic acid derivative by several conventional methods including dilute aqueous potassium permanganate or even periodic acid. This latter reagent must be carefully limited to avoid oxidative cleavage of the D-ribityl side chain. For this periodic acid procedure, 225 mg. of periodic acid in one ml. of water was added dropwise to a cold saturated solution containing 40 mg. of formylriboflavin. With stirring, the carboxyflavin precipitated from solution and was collected by filtration, rinsed with a small quantity of cold water, and dried to give 25 mg. (60%).

8-Formylriboflavin 5′-Phosphate (VIII).

Formylriboflavin (1 g.) was phosphorylated with chlorophosphoric acid essentially as described for the phosphorylation of riboflavin (14) and typical analogs (15). The crude ester obtained was purified by elution from DEAE-cellulose (chloride) as the hydrated lithium salt exactly as described for purification of FMN (16). After dissolving in methanol and precipitating with acetone, 0.7 g. (55%) of dried phosphate ester was obtained.

8-Formylriboflavin-Adenine Dinucleotide (IX).

Formylriboflavin 5′-phosphate (0.5 g.) was condensed in anhydrous pyridine with 0.5 g. of 4-morpholine *N,N′*-dicyclohexylcarboxyamidinium 5′-phosphoromorpholidate as described for the

synthesis of FAD (17) and analogs (7,15) and similarly purified for 0.25 g. (30%) of the hydrated dilithium salt. The compound exhibits a value near 3.5 for the ratio of absorbance at 260 mμ/-450 mμ; fluorescence is optimal near *p*H 3.

REFERENCES

(1) This work was supported in part by Research Grant AM-04585 from the National Institute of Arthritis and Metabolic Diseases, U.S.P.H.S., and in part by funds made available from the State University of New York.

(2) R. Kuhn, F. Weygand, and E. F. Möller, *Chem. Ber.*, **76**, 4044 (1943).

(3) D. B. McCormick, C. Arsenis, and P. Hemmerich, *J. Biol. Chem.*, **238**, 3095 (1963).

(4) E. E. Haley and J. P. Lambooy, *J. Am. Chem. Soc.*, **76**, 5093 (1954).

(5) R. D. Faulkner and J. P. Lambooy, *J. Med. Chem.*, **9**, 495 (1966).

(6) D. B. McCormick, *J. Heterocyclic Chem.*, **4**, 629 (1967).

(7) D. B. McCormick and G. E. Opar, *J. Med. Chem.*, **12**, 333 (1969).

(8) P. Hemmerich, B. Prijs, and H. Erlenmeyer, *Helv. Chim. Acta*, **42**, 2164 (1959).

(9) This and subsequent trivial names for the riboflavin analogs indicate that the original 8-methyl of riboflavin has been replaced by another function, *e.g.* formyl.

(10) P. Hemmerich, A. Ehrenberg, W. H. Walker, L. E. G. Eriksson, J. Salach, P. Bader, and T. P. Singer, *F. E. B. S. Letters*, **3**, 37 (1969).

(11) D. W. Mathieson, Ed., "Nuclear Magnetic Resonance for Organic Chemists," Academic Press, Inc., New York and London, 1968.

(12) F. J. Bullock and O. Jardetsky, *J. Org. Chem.*, **30**, 2056 (1965).

(13) R. Kuhn, H. Rudy, and T. Wagner-Jauregg, *Ber.*, **66**, 1950 (1933).

(14) L. A. Flexser, U. Montclair, and W. G. Farkas, U. S. Patent 12,610,176 (1952).

(15) W. Föry and P. Hemmerich, *Helv. Chim. Acta*, **50**, 1766 (1967).

(16) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).

(17) J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 3756 (1958).

Received October 16, 1969

Ithaca, New York 14850