Synthesis and Biological Activity of 7-Methyl-8-bromo-10-(1′-D-ribityl)isoalloxazine, an Analog of Riboflavin[1] (36908)

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Dichlororiboflavin (7,8-dichloro-10-(1′-D-ribityl)isoalloxazine) (Fig. 1, II) was the first antagonist of riboflavin (Fig. 1, I) to be described (1). It was found to temporarily inhibit *Staphlococcus aureus* and *Streptobacterium plantarum* P-32, neither of which require an exogenous source of riboflavin. Later it was shown (2) that the compound was essentially inert for *Lactobacillus casei*, an organism dependent on an exogenous source of riboflavin. The findings for *L. casei* were confirmed and it was also shown that the compound was inert for the rat (3).

Two analogs of riboflavin whose structures did not depart so extensively from the structure of riboflavin were synthesized (4) for biological evaluation. These two analogs were 7-chloro-8-methyl-flavin (7-chloro-8-methyl-10-(1′-D-ribityl)isoalloxazine) (Fig. 1, III) and 7-methyl-8-chloro-flavin (7-methyl-8-chloro-10-(1′-D-ribityl)isoalloxazine) (Fig. 1, IV). It was found that 7-chloro-8-methyl-flavin is a potent, reversible antagonist of riboflavin in *L. casei* and in the rat. The growth response in the riboflavin-deficient rat when small quantities of this analog are given, was approximately equal to that caused by the administration of riboflavin when the latter was given in quantities equal to one-half the quantities of analog used. Larger amounts are lethal to the rat. This compound is, therefore, the most potent vitamin-like analog of riboflavin in terms of growth of the rat and it is also the most potent antagonist of riboflavin in terms of its lethal properties (5). During the administration of this flavin, the riboflavin-deficient animals recover from the visually observable signs of the deficiency (6). The isomeric 7-methyl-8-chloro-flavin was found to be the most potent reversible antagonist of riboflavin for *L. casei* to have been described. It is able to produce only a small growth response and only moderate improvement in the animals appearance when large amounts are given to rats (3).

The biological properties of 7-chloro-8-methyl-flavin prompted us to synthesize and study the biological properties of 7-bromo-8-methyl-flavin (7-bromo-8-methyl-10-(1′-D-ribityl)isoalloxazine (Fig. 1, V). This analog was found to possess approximately 50% of the potency of 7-chloro-8-methyl-flavin as a reversible antagonist of riboflavin for *L. casei*. It possessed the same potency for growth of the riboflavin-deficient rat as shown by 7-chloro-8-methyl-flavin but it was substantially less toxic (7).

The remarkable properties of the above three analogs of riboflavin possessing single atoms of halogen as replacement for a methyl group in riboflavin made it appropriate for us to synthesize and study the properties of the remaining 7-methyl-8-bromo-flavin (7-methyl-8-bromo-10-(1′-D-ribityl)isoalloxazine (Fig. 1, VI).

Materials and Methods. Chemical. *p*-Nitrotoluene was converted as described (8) to 2-bromo-4-nitrotoluene,[2] mp 78–79°[3] [Lit. (8) gives 75–76°] in 87% yield. The nitro-

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[2] The material was found to be pure by gas chromatography.

[3] All melting points determined on calibrated thermometers.

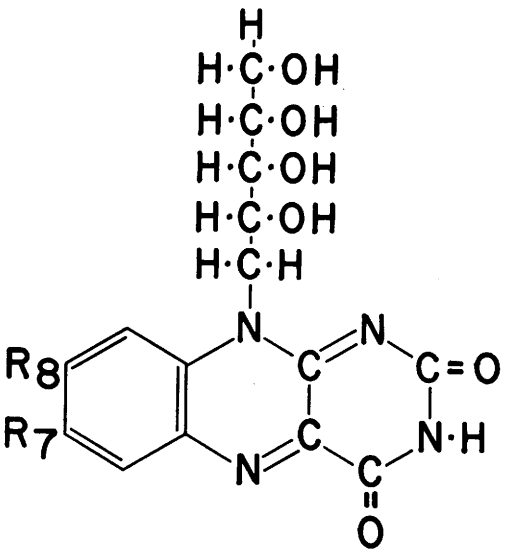

FIG. 1. Basic isoalloxazine or flavin structure.

	Trivial name	R_7	R_8
I	Riboflavin	CH_3–	CH_3–
II	7,8-Dichlororiboflavin	Cl–	Cl–
III	7-Chloro-8-methyl-flavin	Cl–	CH_3–
IV	7-Methyl-8-chloro-flavin	CH_3–	Cl–
V	7-Bromo-8-methyl-flavin	Br–	CH_3–
VI	7-Methyl-8-bromo-flavin	CH_3–	Br–

toluene was reduced[4] to 3-bromo-4-methylaniline, bp 129°/10 mm Hg, 135–137°/13 mm Hg [Lit. (9) gives 254–257°/atm] in 92% yield with iron and hydrochloric acid by a procedure described for a related compound (10). The aniline was acetylated with acetic anhydride to 3-bromo-4-methylacetanilide, mp 115–116° [Lit. (11) gives 113°] in 68% yield by a procedure described for a related compound (12). The acetanilide was converted to 3-bromo-4-methyl-6-nitroacetanilide, mp 122–124° [Lit. (13) gives 120°] in 59% yield by a procedure described for a related compound (12). The nitroacetanilide was converted to 3-bromo-4-methyl-6-nitroaniline, mp 166–168° [Lit. (13) gives 165°] in 84% yield by a procedure described for a related compound (12). The nitroaniline was converted by a procedure used for an isomeric compound (7) to 2-bromo-4-chloro-5-nitrotoluene, mp 66–67° (*n*-hexane) in 74% yield as bright yellow crystals.[2] (Calcd for: C, 33.6%; H, 2.0%; Br, 31.9%; N, 5.6% Found: C, 33.7%; H, 2.1%; Br, 31.5%; N, 5.4%). The 2-bromo-4-chloro-5-nitrotoluene was converted by a procedure used for a related compound (4) to 3-bromo-4-methyl-6-nitro-*N*-D-ribitylaniline, mp 171–172° (methyl alcohol) in 52% yield as bronze colored crystals. (Calcd for: C, 39.5%, H, 4.7%; Br, 21.9%; N, 7.7%. Found: C, 39.3%; H, 4.5%; Br, 21.6%; N, 7.8%). The above ribityl-aniline was converted by the procedure used for its isomeric analog (7) to 7-methyl-8-bromo-10-(1′-D-ribityl)isoalloxazine (7-methyl-8-bromo-flavin) mp 262° (dec.) in 83% yield following recrystallization by the hydrochloric acid procedure described (7) as orange crystals. (Calcd for: C, 43.6%; H, 3.9%; Br, 18.1%; N, 12.7%. Found: C, 43.9%; H, 4.1%; Br, 18.0%; N, 12.4%).

Biological. The procedures described for the study of the 7-methyl-8-chloro-flavin (3) in the metabolism of *L. casei* were used for the study of the analog 7-methyl-8-bromo-flavin. The need for separate autoclaving of the flavin solution and the basal medium was because when mixed, the sterilization procedure caused the analog to be reversibly reduced to a red, stable semiquinine (3).

For the animal studies, 120 female weanling rats of the Wistar strain,[5] weighing 35 to 40 g, were housed individually in wire-bottomed cages and the conditions under which they were maintained have been described before (14). The animals were fed the riboflavin-deficient diet described before (15) until they were deficient. The animals were weighed at weekly intervals, and an animal was designated deficient when it had failed to gain 6 g in weight during a period of 14 days. As the animals became deficient they were distributed at random into eight groups until each group contained 10 or 11 rats. The daily supplements are described in Table I. All flavin supplements were given by stomach tube as a solution or suspension in 0.5 ml of 6% gum acacia solution, each day

[4] Reduction could also be accomplished in 62% yield by the use of Ni and hydrogen. Reductions with Pt and hydrogen led to excess uptake of the latter and a product which was difficult to purify.

[5] C F N rats, Carworth, Inc., New City, NY.

TABLE I. Growth of Rats Receiving Analog, Riboflavin, or Analog Plus Riboflavin.

Group	Daily supplement	Improved appearance (%)[a]	Wt gained (g)[b]		
1	Vehicle	0	7 ± 2		
				(0.157)[c]	
2	20 μg A[d]	50	11 ± 2		
				(0.335)	
3	50 μg A	50	14 ± 2		(0.030)[e]
				(0.031)	
4	250 μg A	82	21 ± 2		
				(0.023)	
5	500 μg A	80	27 ± 2		
				(0.283)	
6	1000 μg A	82	30 ± 2		
7	10 μg Rb	100	72 ± 4		
				(0.009)	
8	10 μg Rb + 500 μg A	100	52 ± 6		
6-1[f]	1000 μg A; 42 days	80	38 ± 5		(6 vs 6-1; $p = 0.130$)
				(0.008)	
6-2[g]	1000 μg A; 42 days 2000 μg A; 28 days	100	63 ± 5		(6 vs 6-2; $p = 0.0001$)

[a] Percentage of animals in the Group which showed improved appearance or improvement in the visually observable signs of riboflavin deficiency.

[b] Net weight gain for 28 day test period ± an estimate of the standard error of the mean.

[c] Probability relation for difference of means of two groups in lines immediately above and below the values in parentheses.

[d] A, analog; Rb, riboflavin.

[e] Probability relation for difference of means between Groups 1 and 3. The p value for all differences between Group 1 and Groups of higher number would be smaller than the one given. For Group 1 vs Group 2, p is 0.157, of course.

[f] Animals in Group 6 continued at 1000 μg/day for an additional 14 days.

[g] Animals in Group 6-1 changed from 1000 μg/day to 2000 μg/day and continued for an additional 28 days.

for 28 days, immediately before the animals were fed.

Molar extinction coefficients were determined from the optical densities determined at wavelengths associated with the absorption maxima and minima. Measurements were made on a Beckman Model DU Spectrophotometer equipped with a Gilford photometric indicator unit (model 220) with combined lamp source stabilizer and amplifier.

The analog 7-methyl-8-bromo-flavin was chromatographed by the descending techniques as described before (16) using the upper layer of a *n*-butanol, water and acetic acid mixture (4:5:1).

Results. The new flavin 7-methyl-8-bromoflavin is devoid of vitamin-like or riboflavin-antagonistic activity for *L. casei*. The analog was tested for vitamin-like properties up to concentrations of 400 μg/tube. Antagonistic activity was tested up to the same concentration of analog in the presence of 0.15 and also 0.3 μg/tube of riboflavin. The analog does, however, provide a small stimulus for the metabolism of this organism when it is made available in the presence of a small amount of riboflavin in the ratio of approximately 1000:1.

The results of the animal studies are so clearly shown in Table I that little comment

is required. The analog caused a growth response at all quantities given although the response was not statistically different from that of the riboflavin-deficient Group until 50 μg/day were administered. The animals showed improvement in their appearance in response to all quantities of the analog administered. When it was found that 1000 μg/day of the analog caused growth and improved appearance and was not lethal during a 28 day test period, the same quantity of analog was administered for an additional 14 days. This caused increased growth but no further improvement in appearance. After these animals had been given 1000 μg/day of the analog for 42 days the quantity of the analog was increased to 2 mg/day and administered an additional 28 days. Thus, after 70 days and the ingestion of a total of 98 mg of the analog, the animals were essentially indistinguishable from those in Groups 7 and 8. No animal died as a result of the administration of the analog.

The molar extinction coefficients of the new flavin 7-methyl-8-bromo-flavin are given with those determined for riboflavin and the related 7-methyl-8-chloro-flavin.

Riboflavin:
Maxima: 267 nm, 33,200; 373 nm, 10, 650; 445 nm, 12,250
Minima: 304 nm, 1200; 399 nm, 6900

7-Methyl-8-chloro-flavin:
Maxima: 267 nm, 31,500; 366 nm, 9650; 444 nm, 12,350
Minima: 302 nm, 1700; 395 nm, 6050

7-Methyl-8-bromo-flavin:
Maxima: 267 nm, 31,690; 369 nm, 9880; 445 nm, 12290
Minima: 301 nm, 1680; 400 nm, 6985

When riboflavin and 7-methyl-8-bromo-flavin were chromatographed simultaneously, the R_f value of the former was 0.29 and of the latter 0.39.

Discussion. The observed biological activities of the new analog of riboflavin were not what we had considered to be reasonably predicted. We had found 7-chloro-8-methyl-flavin (III) to be a potent inhibitor of riboflavin in *L. casei*. In the rat it possessed 50% of the growth promoting potency of riboflavin and it caused complete recovery of the animal's riboflavin-deficiency appearance. Large quantities were lethal for the animal. When the 7-position was occupied by a bromine atom rather than a chlorine atom, as in 7-bromo-8-methyl-flavin (V), the potency of the compound as an antagonist of riboflavin in *L. casei* was reduced. In the rat toxicity was substantially reduced while its growth-promoting and appearance-improving potency were unchanged. Since 7-methyl-8-chloro-flavin (IV) had been found to be a very potent antagonist of riboflavin in *L. casei*, and in the rat a low potency stimulus for growth and improved appearance and of low toxicity, we anticipated that the 7-methyl-8-bromo-flavin (VI) would be a somewhat less but still potent antagonist in *L. casei*, and in the rat to be of low potency as a growth stimulus and a stimulus for improved appearance and of still lower toxicity. We have found, however, that 7-methyl-8-bromo-flavin is devoid of any biological activity for *L. casei*, and while it does not possess antagonistic activity against riboflavin, in the presence of riboflavin it provides an exceedingly small stimulus. Its activity is almost exactly that shown by 7,8-dichlororiboflavin (II) (3). For the rat this new analog was found to be nearly identical to 7-methyl-8-chloro-flavin (IV) as a growth stimulus and it was found to be nontoxic at all levels used. The new flavin was found to possess considerable potency with respect to its ability to improve the visually observable signs of riboflavin deficiency in the rat. Since 10 μg of riboflavin plus 500 μg of analog/day (Group 8) did not cause the animals to grow as well as 10 μg of riboflavin/day alone, the analog possesses inhibitory properties; it interferes with the normal utilization of riboflavin.

When a riboflavin-deficient rat is given riboflavin, it increases its food consumption, improves its food utilization, increases in weight and recovers its normal appearance. The first two and the last responses are due to riboflavin, the third is due to the first two but when adequate food is available to the animal these four responses are intimately linked. It was found that the same relationship among these responses existed when

7-chloro-8-methyl-flavin (III) was given to a riboflavin-deficient rat (6, 15). In the case of 7-methyl-8-bromo-flavin (VI) we find that when adequate food is available to the animal, administration of the flavin provides only a small stimulus to eat and, therefore, the animal gains weight very slowly. The influence on food utilization was not studied but it would be insufficient to play a major role in weight gain. Judging from these findings the flavin has little riboflavin-like activity. The greatly improved appearance of the animals suggests that the new flavin has considerable riboflavin-like activity.

These findings provide evidence that the mechanisms stimulating food consumption and improved appearance which always appear to be intimately related in the recovery process may be sufficiently different so that the processes can be shown to produce independent responses. We are pursuing these studies with new analogs of riboflavin.

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