(5 ml) was refluxed for 0.5 h. Evaporation and recrystallization from MeOH–Me_2CO gave 0.35 g (54.3%) of **7**·HBr as colorless crystals: mp >300 °C. Anal. ($C_{14}H_{19}NO$·HBr) C, H, N.

Compound **8**·HBr and **9**·HBr were obtained by similar O-demethylation of **5** and **6** with 48% HBr in 85 and 80% yield, respectively. **8**·HBr had mp >300 °C (from MeOH). Anal. ($C_{15}H_{21}NO$·HBr) C, H, N. **9**·HBr had mp 294–295 °C (from MeOH). Anal. ($C_{15}H_{21}NO$·HBr) C, H, N.

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(6) The α and β designations are with reference to the hydroaromatic ring. The stereospecificity of the hydrogenation of **4** may be caused by an anchoring effect of the amino group in the former case, giving α isomer, and the steric hindrance of the solvated ammonium cation in the latter case, giving β isomer.[2]
(7) The difference in the *C*-methyl chemical shift between the α-methyl and β-methyl isomers would be the result of a change in environment of the *C*-methyl from a position above the aromatic ring to one away from the aromatic ring.
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Synthesis and Inhibition Analysis of 2(4)-Imino-4(2)-amino-2,4-dideoxyriboflavin, a Dual Antagonist of Riboflavin and Folinic Acid[1a]

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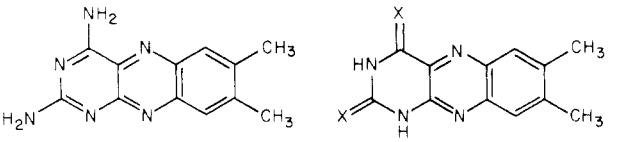
The synthesis of the 2,4-diamino analogue of riboflavin is described. Inhibition analysis in a microbial assay system indicated that this compound has a weak antifolate activity that could be overcome with a minimal amount of folinic acid, but at higher concentrations both folinic acid and riboflavin were required for the reversal of its inhibitory effect.

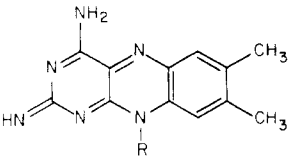
Earlier work in this laboratory, directed at the synthesis of antimetabolites designed to incorporate some of the structural features of two vitamin analogues into a single molecule,[2] led to the synthesis of 2,4-diamino-7,8-dimethylbenzo[*g*]pteridine[3] (**1**) which (in one of its unstable tautomeric forms, **1a**) may be regarded as the 2,4-diimino analogue of lumichrome, the aglycon of riboflavin. This compound was found to be a potent growth inhibitor of *Lactobacillus leichmannii*,[4] with an I_{50} of 0.1–0.2 μg/ml. The inhibition could be reversed competitively with folinic acid alone over a 200-fold concentration range, while at higher concentrations of the inhibitor, both folinic acid and riboflavin were required for reversal.[5] Thus, compound **1** appeared to act as a typical 2,4-diaminopyrimidine-fused heterocyclic inhibitor of dihydrofolate reductase[2,4] but, at high concentrations, also as an antimetabolite of vitamin B_2. Unfortunately, the poor solubility and tissue absorption properties of compound **1** severely limited its in vivo evaluation as a chemotherapeutic agent, although preliminary tests using various transplanted tumors in mice indicated that **1** may possess significant antitumor activity.[6,7]

It was felt that introduction of alkyl or hydroxyalkyl side chains in the N_{10} position of **1** might lead to compounds with better solubility and/or membrane transport properties. Such compounds, having flavin-type structures (e.g., **2**), would bear a greater structural resemblance to riboflavin, while, on the other hand, they would be less analogous to the 2,4-diaminopteridine-type antifolates; therefore, they might be expected to act as more effective antagonists of vitamin B_2 but to show perhaps less activity as inhibitors of dihydrofolate reductase. Initially, we attempted to synthesize the desired N_{10}-substituted derivatives of **1** by various modifications of our general method[3] for the synthesis of 2,4-dideoxyalloxazines (including **1**), i.e., the condensation of appropriately substituted 5,6-diaminopyrimidines with dimeric 4,5-dimethyl-*o*-benzoquinone. Although this method was later successfully employed in the synthesis of 2-imino-2-deoxyflavins (as well as in a new synthesis of riboflavin) by other investigators,[8,9] its application to the synthesis of 2(4)-imino-4(2)amino-2,4-dideoxyflavins invariably led to condensation products which were difficult to purify. Attempts to introduce an alkyl side chain at the N_{10} position of compound **1** by direct alkylation resulted in substitution at the N_1 position.[10] Subsequently, a series of 6-(*N*-alkyl-*N*-arylamino)pyrimidines was prepared as "open chain" analogues and potential synthetic intermediates of the desired N_{10}-substituted 2,4-dideoxyisoalloxazines.[11] A method for the conversion of such compounds derived from uracil to the corresponding isoalloxazines, including riboflavin, was recently reported by Yoneda et al.[12,13]

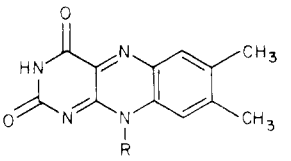


1

1a, X = NH
lumichrome, X = O



2

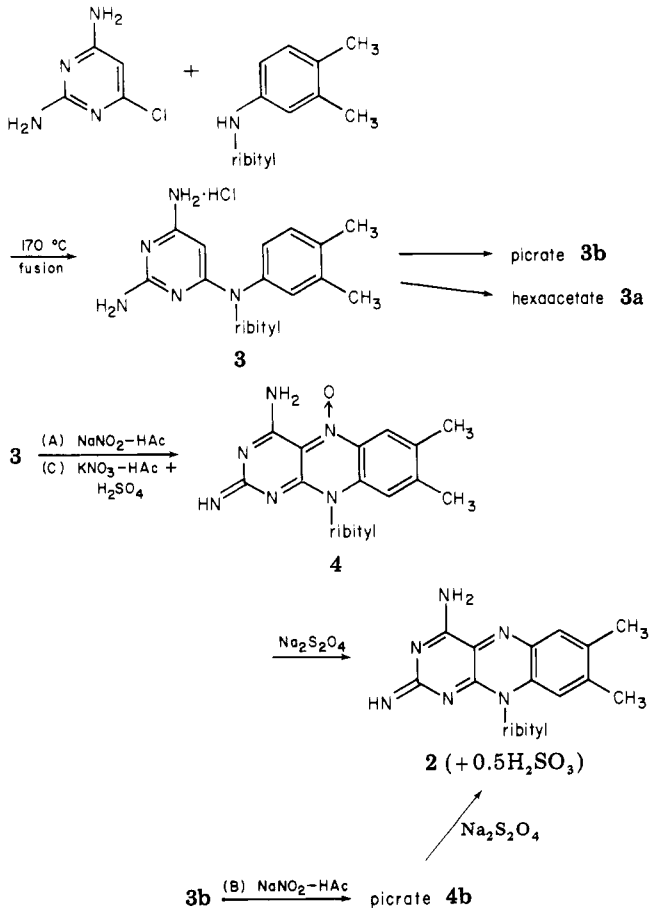


riboflavin

R = $CH_2(CHOH)_3CH_2OH$

We found that the latter method, with some modifications, could be applied to the synthesis of 2(4)-imino-4(2)-amino-2,4-dideoxyriboflavin (**2**) (see Scheme I). Thus, 2,4-diamino-6-chloropyrimidine was allowed to react with

Scheme I



3,4-dimethyl(*N*-ribityl)aniline by fusion at 170 °C for 1–2 h to give 2,4-diamino-6-(*N*-ribityl-3,4-dimethylanilino)-pyrimidine (**3**) in 75% yield. Due to the difficulties encountered in the purification of **3** for elemental analysis, this compound was identified as the hexaacetate **3a** (obtained in quantitative yield by acetylation of **3** with acetic anhydride and pyridine) and, alternatively, as the picrate salt **3b**. Treatment of **3** with sodium nitrite in acetic acid (method A) gave 50% crude product, tentatively identified as 2(4)-imino-4(2)-amino-2,4-dideoxyriboflavin N_5-oxide (**4**) on the basis of its characteristic ultraviolet spectrum and by its reduction to **2** (see below). Although the product from method A was not purified, the analytically pure *N*-oxide was obtained in 89% yield directly in the form of its picrate salt **4b** when the same reaction was applied to **3b** (method B). The hexaacetate **3a** could not be converted to the corresponding *N*-oxide under the same reaction conditions.

Alternatively, compound **4** was obtained by treatment of **3** with potassium nitrate in acetic acid, in the presence of sulfuric acid (method C), and purified after conversion to the picrate **4b**. Thus, either nitrosation (at least, in the form of picrate) or nitration of **3** led to the ring-closed *N*-oxide, similarly as in the case of the analogous riboflavin synthesis of Yoneda et al.[12] The *N*-oxide **4**, either as the free base or as the picrate (**4b**), was then reduced with sodium dithionite to give the desired product, 2(4)-imino-4(2)-amino-2,4-dideoxyriboflavin (**2**), in the form of its sulfite salt.

Inhibition Analysis. In the *L. leichmanii* assay routinely used in our laboratory,[3-5,15] compound **2** produced full inhibition of growth at 20 μg/ml and half-maximal inhibition at 7 μg/ml concentration. At 20–60 μg/ml, the inhibition could be partially reversed with 0.02 μg/ml of folinic acid (inhibition index[14,15] $[I/S]_{50}$ = 6000). At higher concentrations of **2**, virtually no reversal of the inhibition could be obtained with folinic acid alone, but both folinic acid and riboflavin were required for reversal. Thus, at a concentration of 150 μg/ml the inhibitory effect of **2** could be reversed in the presence of 0.02 μg/ml of folinic acid with 250 μg/ml of riboflavin, and the inhibition index (i.e., I/S at 50%) for riboflavin was 1.5.

These results indicate that **2** has a weak antifolate activity that can be overcome with a minimal amount of folinic acid; but at higher concentrations, it shows additional inhibitory effect that can be reversed with an approximately equimolar amount of riboflavin. This type of antagonism between the two close structural analogues, compound **2** and riboflavin, may be due to competition either for cellular uptake or for binding at an enzyme site. However, if riboflavin were to act as a reversing agent merely by interfering with the uptake of **2**, then riboflavin alone would reverse the inhibition without the required presence of a minimal amount of folinic acid (which can act only intracellularly). Therefore, it seems more likely that the antagonism is at an enzyme site; i.e., compound **2** appears to act under these conditions as an antimetabolite of riboflavin.

The significantly lower growth inhibitory activity of **2** as compared to **1** in *L. leichmannii* (a system particularly sensitive to antifolates)[4] is consistent with its much weaker antifolate activity. This substantial loss of antifolate activity which results from the introduction of a ribityl side chain at the N_{10} position of the benzo[*g*]pteridine ring system of **1** may be due to (1) loss of hydrophobicity of the molecule as a whole, (2) lack of "bulk tolerance", or the presence of a hydrophobic region[16] of the enzyme (dihydrofolate reductase) at the site corresponding to the position of the ribityl group, or (3) shift of the π electrons and change from the normal tautomeric structure of the 2,4-diaminopyrimidine moiety of the typical dihydrofolate reductase inhibitors to that of the flavins. On the other hand, these changes have led to a new derivative which, at least from a structural point of view, is a "classical antimetabolite"[2] of riboflavin. Whether the apparent antiriboflavin activity of **2** can be demonstrated in other (particularly, mammalian) test systems, and whether it is of sufficient interest for potential application in cancer chemotherapy,[17-19] is currently being studied in other laboratories.

Experimental Section

All melting points were taken by the capillary tube method on a Mel-Temp and are uncorrected. Where elemental analyses are indicated only by symbols of the elements, analytical results were within ±0.4% of the theoretical values. Ultraviolet spectra were taken on Gilford 2400 and Beckman DB-G spectrophotometers and IR spectra on a Beckman IR-8 spectrophotometer in compressed potassium bromide disks. ^{1}H NMR spectra were taken on a Varian A-60 spectrophotometer and the chemical shifts are expressed as parts per million from tetramethylsilane as an internal standard in the solvent indicated, except with trifluoroacetic acid as solvent in which case tetramethylsilane in chloroform was used as an external standard.

2,4-Diamino-6-(*N*-ribityl-*N*-3,4-dimethylanilino)pyrimidine Hydrochloride (3). A well-ground mixture of 2,4-diamino-6-chloropyrimidine (1.445 g, 0.001 mol) and 6-D-ribitylamino-3,4-dimethylbenzene (2.55 g, 0.001 mol) was fused at 166–170 °C for 1–2 h in a round-bottom flask, and at the end of the reaction 30 ml of ethanol was added. The ethanolic solution was evaporated to dryness to yield a colorless foam. In addition to the desired product, some starting material could be detected by TLC. The desired product (**3**) was purified by chromatography on a silica gel column using methanol–CH_2Cl_2–$CHCl_3$ (1:2:2) as eluent: yield 3.0 g (75%). This compound was identified in the form of

the hexaacetate **3a** which was obtained in quantitative yield by treatment with acetic anhydride and pyridine at room temperature followed by recrystallization from alcohol: mp 165–166 °C; UV λ_{max}^{EtOH} 290 nm (ϵ 10700); IR (KBr) 1750 [$CH_3C(=O)$-] and 1215 cm^{-1} (ester); NMR ($CDCl_3$) δ 1.92 (6 H, s, CH_3CO), 2.02 (3 H, s, CH_3CO), 2.10 (3 H, s, CH_3CO), 2.14 (3 H, s, CH_3CO), 2.28 (6 H, s, CH_3), 2.52 (3 H, s, CH_3CO), 4.05–4.36 (4 H, b, CH_2), 5.16–5.54 (3 H, b, CH), 6.84–7.27 (4 H, m, CH), 10.10 (1 H, b, NHCO), 10.40 (1 H, b, NHCO). Anal. (**3a**, $C_{29}H_{37}N_5O_{10}$) C, H, N.

2,4-Diamino-6-(*N*-ribityl-*N*-3,4-dimethylanilino)pyrimidine Picrate (3b). To a solution of **3** (3.99 g, 0.01 mol) in 50 ml of water was added saturated picric acid solution. The yellow precipitate formed was collected and recrystallized from ethanol to give 5.5 g (93%): mp 206–207 °C; UV λ_{max}^{EtOH} 280 nm (ϵ 19520), 358 (15590), and 390 (sh) (11410); IR (KBr) 1335 cm^{-1} (NO_2); NMR (Me_2SO-d_6) δ 2.22 (6 H, s, CH_3), 3.47–4.35 (11 H, b, ribityl), 4.91 (1 H, s, pyrimidine), 7.20–7.66 (6 H, m, aromatic and amino), 8.85 (2 H, s, picrate). Anal. (**3b**, $C_{23}H_{28}N_8O_{11}$) C, H, N.

2(4)-Imino-4(2)-amino-2,4-dideoxyriboflavin 5-*N*-Oxide (4). Method A. Sodium nitrite (0.385 g, 0.00557 mol) in 5 ml of water was added slowly to an acetic acid (25 ml) solution of **3** (2.0 g, 0.005 mol). The solution immediately turned deep red; it was stirred 7–8 h at room temperature, and then the solvents were evaporated to dryness in vacuo. The residue was suspended in water and then neutralized with ammonia solution. The red precipitate formed was collected and recrystallized from hot water to yield 1.0 g (50%) of still impure **4**: UV λ_{max}^{EtOH} 274, 376, and 480 nm.

Method B. To a solution of the picrate of **3** (**3b**) (5.92 g, 0.01 mol) in acetic acid (40 ml), sodium nitrite (0.76 g, 0.011 mol) in 5 ml of water was added dropwise and then the reaction mixture was stirred for 2 h at room temperature. The solvents were evaporated; then water was added to the residue to induce crystallization: yield 5.4 g (87%) of the picrate **4b**. This was recrystallized from hot water: mp 180–183 °C; UV λ_{max}^{EtOH} 223 nm (ϵ 28000), 278 (45240), 372 (23570), 482 (11960), and 504 (sh) (10940); IR (KBr) 3375 cm^{-1} (NH_2 and/or OH). Anal. (**4b**, $C_{23}H_{25}N_9O_{12}$) H, N; C: calcd, 44.58; found, 44.10.

Method C. A mixture of **3** (1.0 g, 0.0025 mol), potassium nitrate (500 mg, excess), three drops of sulfuric acid, and 15 ml of acetic acid was heated at 105–110 °C for 30 min; then the solvent was evaporated to dryness in vacuo. Water was added to the residue and the red precipitate formed was collected to yield 0.4 g (40%) of **4**, which was converted to the picrate **4b**, identical with that obtained by method B (see above).

2(4)-Imino-4(2)-amino-2,4-dideoxyriboflavin (2) Sulfite. Method A. To a suspension of the *N*-oxide **4** (3.0 g, 0.0077 mol) in 40 ml of water and 5 ml of strong ammonia solution, sodium dithionite (1.34 g, 0.0077 mol) in 10 ml of water was added dropwise; then the reaction mixture was stirred for 4 h. The yellowish precipitate formed was filtered and recrystallized from acetic acid–water (9:1) and then from hot water to yield 2.7 g (80%) of brown needles: mp >300 °C; UV $\lambda_{max}^{H_2O}$ (pH 6.1) 226 nm (ϵ 17880), 276 (31250), 380 (7800), and 455 (9510); IR (KBr) 3340 and 3190 cm^{-1} (NH_2 and/or OH); NMR (TFA) δ 2.17 (3 H, s, CH_3), 2.32 (3 H, s, CH_3), 3.83 (2 H, br s, ribityl), 4.01 (2 H, br s, ribityl), 4.46 (1 H, br s, ribityl), 5.03 (2 H, br s, ribityl), 7.73 (2 H, s, aromatic). Anal. ($C_{17}H_{22}N_6O_4 \cdot 0.5H_2SO_3 \cdot H_2O$) C, H, N, S.

Method B. To a solution of the picrate **4b** (3.045 g, 0.005 mol) in hot water (100 ml) was added $Na_2S_2O_4$ until the reddish solution became yellow. The reaction mixture was stirred for 2 h at room temperature. The brown precipitate formed was collected (2.2 g, 73%) and recrystallized from water to give **2** in the form of the sulfite salt, as above.

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(1) (a) A preliminary report of this work was presented before the Medicinal Chemistry Division, 170th National Meeting of the American Chemical Society, Chicago, Ill., August 1975, MEDI 53. (b) Abstracted in part from a portion of the dissertation submitted by C.K.C to the faculty of the State University of New York at Buffalo, in May 1975, in partial fulfillment of the requirements for a Ph.D. degree.
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