Biosynthesis of Riboflavin
Preparation of Phosphorylated Pyrimidine Intermediates

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Riboflavin, Biosynthesis, 2,5-Diamino-6-ribitylamino-4(3H)-pyrimidinedione 5'-Phosphate, 5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-Phosphate, Pyrimidine Deaminase

2-Amino-5-nitro-6-(1'-D-ribityl)amino-4(3H)-pyrimidinone (6a) was converted to the dimethoxytrityl derivative, which was subsequently acetylated. The trityl residue was then removed, and the compound was phosphorylated by the cyanethyl method. Removal of the protecting groups yielded 2-amino-5-nitro-6-(1'-D-ribityl)aminopyrimidinone 5'-phosphate (11a). Catalytic hydrogenation yielded the labile 2,5-diamino-6-(1'-D-ribityl)aminopyrimidinone 5'-phosphate (3a) which is the substrate for a deaminase from yeast. 5-Amino-6-(1'-D-ribityl)aminopyrimidinedione 5'-phosphate (3b) was prepared by an analogous sequence of reactions. It was further converted to 6,7-dimethyl-8-(1'-D-ribityl)lumazine 5'-phosphate (12b). The phosphoric acid esters prepared by the present procedures contained about 20% of an undesired isomer as a consequence of insufficient selectivity of the tritylation reaction.

Introduction

The biosynthesis of riboflavin in yeasts is summarized in Fig. 1 (for review see Ref. [1, 2]). The pyrimidine intermediates 3a and 3b were first observed in dephosphorylated form in the culture medium of riboflavin deficient mutants of the yeast Saccharomyces cerevisiae [3–5]. Later enzymatic studies suggested that the actual biosynthetic inter-

![Fig. 1. Biosynthesis of riboflavin in yeast.](image-url)
mediates should be the phosphoric acid esters 3a and 3b. However, due to the poor stability of these compounds, the arguments were based on indirect evidence [6—9].

This paper describes the chemical synthesis of the phosphoric acid esters 3a and 3b. The conversion of 3a prepared by this procedure to 3b by cell extracts from the yeasts Candida guilliermondii and Saccharomyces cerevisiae has been reported elsewhere [10, 11]. Some of the data have been reported in abstract form [12].

**Results and Discussion**

The synthetic reactions used in this study are summarized in Fig. 2. Briefly, the synthesis started from 5-nitro-6-ribitylaminopyrimidine derivatives (6). Initial attempts to use the 5-nitroso derivatives were unsuccessful because the compounds were not sufficiently stable under the experimental conditions. Reaction of the nitropyrimidine derivatives 6 with 4,4'-dimethoxytritylchloride yielded the dimethoxytrityl derivatives 7 which were subsequently acetylated. The dimethoxytrityl group of 8 was removed under mild conditions, and the product 9 was phosphorylated by the cyanethyl phosphate procedure [13]. The cyanethyl group and the acetyl groups could be removed by aqueous ammonium hydroxide at room temperature. The resulting phosphoric acid esters were purified by column chromatography on DEAE cellulose. Catalytic hydrogenation with palladium on charcoal yielded the corresponding 5-amino compounds 3.

The 5-aminopyrimidines 3a and 3b are very unstable, and no attempt was made to isolate them in solid form. The solutions were prepared as needed and were used directly in enzyme studies. The enzymatic formation of 3a from 3b, which had been synthesized by the present method, has been reported elsewhere [10, 11].

Reaction of 3b with diacetyl yielded the lumazine phosphate 12b which has been previously prepared in small quantities by enzymatic phosphorylation of 4 using phosphotransferase from carrots [14]. This
compound deserves interest as a structural and functional analogue of riboflavin 5′-phosphate [15].

This study provides the first synthetic approach to the intermediates 3a and 3b in the biosynthesis of riboflavin. However, the method has some shortcomings. The protected synthetic intermediates could not be obtained in crystalline, analytically pure state, and the final products contained about 20% of an isomeric phosphoric acid ester. These problems are closely related and result from the insufficient selectivity of the tritylation reaction. It was shown that the monotrityl compound 7a can react under the experimental conditions with an excess of dimethoxytrityl chloride with ultimate formation of a tris(trityl) product (14). This appears to be due to the tritylation of more than one hydroxyl group in the ribityl side chain, since the model compound 2-amino-6-(2′-(4,4′-dimethoxytrityl)oxyethylamino)-4(3H)-pyrimidinone (13) was resistant against further tritylation under the same conditions. Attempts to increase the selectivity of the tritylation of 6 and avoid the formation of multiply tritylated species by modification of the experimental conditions were unsuccessful, nor was it possible to purify the products by recrystallization because resinous materials invariably formed at elevated temperatures.

Recent studies from this laboratory have shown that isomeric phosphoric acid esters of riboflavin and related compounds can be separated efficiently by reverse phase high performance liquid chromatography (HPLC) [16]. HPLC analysis of the synthetic lumazine 12 showed two well-resolved peaks accounting for 80 and 20% of the total sample (Fig. 3). The major peak was unequivocally identified as the 5′-phosphate by binding to apoflavodoxin from Megasphaera elsdenii [16] using the method of Was-sink and Mayhew [17]. It has been shown earlier that this protein shows very high selectivity for 5′-phosphoric acid esters [16, 18, 19].

HPLC analysis of the phosphorylated pyrimidines 11a (Fig. 4) and 11b likewise showed the presence of
two isomers at a ratio of about 4:1. The major isomer of 11a was isolated in pure form by preparative HPLC; starting from the pure isomer, a sequence of catalytic hydrogenation, enzymatic deamination, and condensation with diacetyl yielded 6,7-dimethyl-8-ribityllumazine 5'-phosphate which was unequivocally identified by HPLC procedures and apoflavodoxin titration [11, 12]. It follows that the chemical synthesis leads to an isomer mixture containing about 80% of the respective 5'-phosphate. The minor isomer present in 11a was identified as the 4'-phosphate by a quantitative study of acid-catalyzed isomerization (P. Nielsen and A. Bacher, unpublished data). Pure 5'-phosphates can be obtained from the isomer mixtures by preparative HPLC procedures [16].

The preparation of isomerically pure phosphate esters of polyol derivatives is generally difficult to achieve. The most notable example is the synthesis of riboflavin 5'-phosphate (FMN). The compound is structurally similar to the compounds reported in this paper and is produced in large amounts for pharmaceutical purposes. Since the first synthesis reported by Kuhn and coworkers [20] in 1936, numerous publications and patents have appeared. However, as reviewed in detail by Scola-Nagelschneider and Hemmerich [19], all known procedures lead to a mixture of products containing at best about 75% of the target compound. Pure 5'-FMN is only available by affinity chromatography [18] or by HPLC separation [16].

It should be noted that 3a produced by the method reported in this paper can be used for enzymatic work without further purification. The contaminating 4'-phosphate is neither a substrate nor an inhibitor of the pyrimidine deaminase from yeast. If necessary, isomer separation is possible by preparative HPLC at the nitropyrimidine (11) or lumazine (12) level [16].

Experimental Section

General methods

Melting points are uncorrected. NMR spectra were recorded with a Bruker WP 200 instrument using CDCl₃ as solvent. Field desorption mass spectra were recorded with a MAT 311 instrument. Visible and ultraviolet spectra were measured with a Zeiss PM 6 photometer. Phosphate was determined after hydrolysis with alkaline phosphatase by the procedure of Eibl and Lands [21]. Thin layer chromatography was performed with precoated silica gel plates (Merck) which were developed with a mixture of chloroform/methanol (9:1, v/v). Dimethoxytrityl derivatives formed yellow spots when the plates were heated at 160 °C. High performance liquid chromatography (HPLC) was performed with a Waters instrument. Columns of Nucleosil 10 C₁₈ (10 μm, 4 × 250 nm) were used. The eluent was 0.1 M ammonium formate pH 3.7 [16]. Pyridine was distilled over calcium hydride and stored over 4 Å molecular sieves. Acetic anhydride was distilled over sodium acetate. Dimethylsulfoxide was stored over 4 Å molecular sieves.

2-Amino-5-nitro-6-ribitylamino-4(3H)-pyrimidinone (6a)

The compound was prepared by a modification of the published procedure [22]. Finely powdered 2-amino-6-chloro-5-nitro-4(3H)-pyrimidinone (10 g, 61 mmol) [22] was added to a solution of 0.13 mol of D-ribitylamine in 280 ml of 25% aqueous ethanol. The mixture was heated at 60 °C for 14 h with stirring. A voluminous precipitate formed. The mixture was set aside overnight. The precipitate was collected by suction, washed with water and dried over P₂O₅. Yield, 11.4 g, 61% based on pyrimidine. Melting point, 253—254 °C (literature [22], 245—246 °C, decomp.).

Analysis calculated for C₉H₁₅N₅O₇
Calcd  C 35.41  H 4.95  N 22.94,
Found   C 35.35  H 5.20  N 22.86.

2-Amino-6-(5'-(4,4'-dimethoxytrityl)ribitylamino)-5-nitro-4(3H)-pyrimidinone (7a)

To a solution of 6a (1.2 g, 3.9 mmol) and 4,4'-dimethoxytritylchloride (2.0 g, 5.9 mmol) in 10 ml of dimethysulfoxide, pyridine (5 ml) was added, and the mixture was stirred at room temperature for 2 h. Chloroform (100 ml) was added, and the solution was washed twice with water. The chloroform phase was dried over anhydrous Na₂SO₄ and filtered. Isopropanol (100 ml) was added, and the volume was reduced to 50 ml by slow evaporation under reduced pressure. The white precipitate formed was collected and dissolved in chloroform (50 ml). 2-Propanol (100 ml) was added, and the volume was reduced by evaporation under reduced pressure until a white precipitate formed, which was collected and dried over P₂O₅. Yield, 1.7 g, 71%. Melting point, 169—172 °C (sinters above 160 °C).

Analysis calculated for C₉₀H₁₃₀N₁₅O₉
Calcd  C 59.30  H 5.47  N 11.53,
Found   C 60.46  H 5.46  N 10.71.
λ\text{max}\text{ in CHCl}_3, 333 \text{ nm (11,300 M}^{-1}\text{ cm}^{-1}). Thin layer chromatography showed the presence of some bis-tritylated material. Recrystallization was not possible due to the rapid formation of resinous products at elevated temperature.

2-Amino-5-nitro-6-(2',3',4'-triacetyl-5'-\(\text{H},3\text{H})\)-pyrimidinedione (8a)

\textbf{Method A.} Freshly distilled acetic acid anhydride (5 ml) was added to a solution of 7a (1.5 g, 2.5 mmol) in 10 ml of dry pyridine. The mixture was kept at room temperature for 1 h. Chloroform (80 ml) was added and the solution was washed 3 times with water. 2-Propanol (150 ml) was added. Partial evaporation under reduced pressure induced the formation of a white precipitate which was collected and dried over P_2O_5. Yield, 1.1 g, 60%.

\textbf{Method B.} A suspension of finely powdered 6a (3.0 g, 9.8 mmol) and dimethoxytritylchloride (4.2 g, 12.4 mmol) in 50 ml of anhydrous pyridine was stirred in a tightly sealed flask until the solid had dissolved (about 1 h). A mixture of acetic anhydride (20 ml) and anhydrous pyridine (20 ml) was added and the solution was set aside at room temperature overnight. Chloroform (100 ml) was added, and the product was isolated as described under Method A. Yield, 5.2 g, 72%. Melting point, 143–150°C (sinters above 140°C).

\textit{Analysis calculated for C}_{36}H_{30}N_{10}O_{12}

\textit{Calcd} C 58.93 H 5.36 N 9.54,

\textit{Found} C 59.83 H 5.37 N 9.09.

λ\text{max} \text{ in CHCl}_3, 330 \text{ nm (15,200 M}^{-1}\text{ cm}^{-1}). The \(^1H\) NMR spectrum indicates 3 acetyl groups (singlets at δ = 1.89, 3.02, and 2.20). The compound could not be recrystallized. Decomposition with formation of resinous materials occurred at elevated temperatures. Thin layer chromatography showed the presence of some double-tritylated material with both procedures reported. This accounts for the excess of carbon and corresponding defect of nitrogen in elemental analysis.

5-Nitro-6-(5'-\(\text{H},3\text{H})\)-pyrimidinedione (8b)

The compound was prepared from 6b [22] as described for 3a. Yield, 70%. White solid, m.p. 119–123°C (sinters above 110°C).

\textit{Analysis calculated for C}_{36}H_{30}N_{10}O_{12}

\textit{Calcd} C 58.85 H 5.21 N 7.63,

\textit{Found} C 58.78 H 5.42 N 6.99.

The \(^1H\) NMR indicates 3 acetyl groups (singlets at δ = 1.90, 1.98 and 2.16). Mass spectrum, m/e = 734.2. λ\text{max} \text{ in CHCl}_3, 324 \text{ nm (11,500 M}^{-1}\text{ cm}^{-1}).

2-Amino-5-nitro-6-(2',3',4'-triacetyl)ribitylamino-4(3H)-pyrimidinedione (9a)

A suspension of 8a (1.3 g, 1.9 mmol) in 50 ml of 80% acetic acid was stirred at room temperature. The solid dissolved rapidly. After 1 h, the solution was evaporated to dryness under reduced pressure. The oily residue was dissolved in 30 ml of chloroform with 5 drops of pyridine. 2-Propanol (100 ml) was added, and the volume was reduced to 30 ml by evaporation under reduced pressure. A gelatinous precipitate formed. It was collected on a Buchner funnel and dried over P_2O_5. The compound could not be recrystallized. Yield, 0.5 g, 61%. Melting point, 225–235°C (sinters above 200°C).

\textit{Analysis calculated for C}_{36}H_{30}N_{10}O_{12}

\textit{Calcd} C 41.77 H 4.91 N 16.24,

\textit{Found} C 41.68 H 5.01 N 15.97.

λ\text{max} \text{ in CHCl}_3, 328 \text{ nm (12,700 M}^{-1}\text{ cm}^{-1}). Mass spectrum, m/e = 432.

5-Nitro-6-(2',3',4'-triacetyl)ribitylamino-2,4(1H,3H)-pyrimidinedione (9b)

The compound was prepared from 8b by the same procedure as 9a. Yield, 76%, white solid, m.p. 95–102°C (sinters above 90°C).

\textit{Analysis calculated for C}_{36}H_{30}N_{10}O_{12}

\textit{Calcd} C 41.67 H 4.66 N 12.96,

\textit{Found} C 42.05 H 4.98 N 12.50.

λ\text{max} \text{ in CHCl}_3, 331 \text{ nm (9,800 M}^{-1}\text{ cm}^{-1}). Mass spectrum, m/e = 433.

2-Amino-5-nitro-6-ribitylamino-4(3H)-pyrimidinone 5'-phosphate (11a)

9a (400 mg, 0.93 mmol) was dissolved in a pyridine solution of 4 mmol cyanoethylphosphate [13] and the solution was brought to dryness under reduced pressure. Anhydrous pyridine (10 ml) was added, and the solution was again brought to dryness under reduced pressure. This step was repeated twice to remove water. The residue was dissolved in 10 ml of anhydrous pyridine, and 1.06 g (5.1 mmol) of dicyclohexylcarbodiimide was added. The mixture was kept in a tightly sealed flask for 2 d. One ml of water was added and after 1 h at room temperature the mixture was brought to dryness under reduced pressure. Ammonium hydroxide (9 M, 50 ml) was added and the mixture left overnight at room temperature. Dicyclohexylurea was removed by filtra-
tion. The solution was extracted twice with 100 ml of CHCl₃. The aqueous solution was brought to dryness under reduced pressure. The residue was dissolved in 30% isopropanol (10 ml) and applied to a column of DEAE cellulose (Whatman DE 52, acetate form, 2.5 x 15 cm). The column was developed with a linear gradient of 0—0.5 M triethylammonium acetate pH 7.0 in 30% isopropanol (total volume, 1 l). Fractions were combined and concentrated to dryness under reduced pressure. The oily residue was dissolved in 20 ml of dry methanol. The solution was poured into 500 ml of absolute ether. The precipitate formed was collected on a Buchner funnel and dried over P₂O₅. Yellow, very hygroscopic solid. Yield, 0.32 mmol, 35% (determined photochemically). 

\[ \lambda_{\text{max}} \text{ at pH 1: 335 nm, 260 nm; } \varepsilon_{335}:\varepsilon_{260} = 6.3. \lambda_{\text{max}} \text{ at pH 7: 335 nm, 259 nm; } \varepsilon_{335}:\varepsilon_{259} = 5.8. \text{ Phosphate content, 0.82 mol per mol of pyrimidine after hydrolysis with alkaline phosphatase. HPLC analysis showed the presence of about 20% 4'-phosphate. Pure 5'-phosphate can be prepared by preparative HPLC [16].] 

5-Nitro-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate (11b)

The compound was prepared in analogy to 11a. The DEAE column was developed with a gradient of triethylammonium acetate 0—0.5 M, pH 4.3. Yield, 40% (determined photometrically). \[ \lambda_{\text{max}} \text{ at pH 1: 324 nm, 278 nm; } \varepsilon_{324}:\varepsilon_{278} = 7.7. \lambda_{\text{max}} \text{ at pH 7: 333 nm, 260 nm; } \varepsilon_{333}:\varepsilon_{260} = 6.0. \text{ HPLC showed the presence of about 20% 4'-phosphate. Pure 5'-phosphate can be prepared by preparative HPLC [16].] 

2,5-Diamino-6-ribitylamino-4(3H)-pyrimidinedione 5'-phosphate (3a)

The compound was prepared as required for enzyme experiments. An aqueous solution of 11a (triethylammonium salt) was hydrogenated in the presence of palladium on charcoal. The solution was freed from the catalyst by passing it through a nitrocellulose filter and used immediately.

5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate (3b)

The compound was prepared as described for 3a and used immediately.

6,7-Dimethyl-8-ribityllumazine 5'-phosphate (12b)

A solution of 80 \( \mu \text{mol} \) 3b in 3 ml of water was adjusted to pH 4 by the addition of acetic acid, and 0.5 ml of diacetyl was added. The solution was gas-

sed with nitrogen and set aside overnight. It was then placed on a column of Dowex 1 (200—400 mesh, acetate form, 11.6 x 0.9 cm). The column was developed with 50 ml of 0.02 M triethylammonium acetate pH 4.3 and subsequently with a linear gradient of 0.02—2.0 M triethylammonium acetate pH 4.3. Green fluorescent fractions were combined. Yield, 50 \( \mu \text{mol} \), 63% (determined photometrically). The solution was brought to dryness under reduced pressure. The oily residue was dissolved in 5 ml of ethanol. The solution was poured into 50 ml of ether. The dark yellow precipitate was collected on a porous glass filter. Phosphate content, 0.99 mol per mol lumazine. \[ \lambda_{\text{max}} \text{ in 0.1 N HCl, 408 nm, 257 nm (shoulder at 275 nm). } \varepsilon_{408}:\varepsilon_{275} = 0.65. \text{ Titration with an excess of apoflavodoxin (courtesy of Prof. S. Ghisla) reduced the fluorescence to 80% of the original value. HPLC showed the presence of about 20% 4'-phosphate. Pure 5'-phosphate can be prepared by preparative HPLC [16].] 

2-Amino-6-(2'(4,4'-dimethoxytrityl)-oxyethylamino)-4(3H)-pyrimidinone (13)

The compound was prepared from 400 mg (1.85 mmol) of 2-amino-6-hydroxyethylamino-5-nitro-4(3H)-pyrimidinone [24] and 750 mg (2.22 mmol) of dimethoxytritylchloride as described for 7a. Yield, 0.25 g, 26.1%, m.p. 269°C.

Analysis calculated for C₂₇H₂₇N₂O₆.

Calcd C 62.66 H 5.22 N 13.53,

Found C 62.55 H 5.33 N 13.52.

\[ \lambda_{\text{max}} \text{ in CHCl₃, 333 nm (14,900 M}⁻¹\text{cm}⁻¹).] 

2-Amino-5-nitro-4-(tris-4,4'-dimethoxytrityl)-ribitylamino-4(3H)-pyrimidinone (14)

A solution of 6a (0.5 g, 1.6 mmol) and 4,4'-dimethoxytritylchloride (3.9 g, 8.9 mmol) in 8 ml of pyridine was set aside for 2 d. Methylene chloride was added. The solution was washed with water. The organic phase was dried, and ethanol was added. Removal of methylene chloride by partial evaporation under reduced pressure produced a white solid, which was precipitated repeatedly by partial evaporation from a methylene chloride/ethanol mixture. Melting point, 174—176°C. This material still contained some bis-tritylated material as shown by thin layer chromatography. It could not be recrystallized.

Analysis calculated for C₃₇H₄₀N₄O₁₃.

Calcd C 71.33 H 5.74 N 5.78,

Found C 69.86 H 5.71 N 5.7.

\[ \lambda_{\text{max}} \text{ in CHCl₃, 332 nm (11,800 M}⁻¹\text{cm}⁻¹). \text{ Treatment with 80% acetic acid yields 6a.} \]
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