Biosynthesis of Riboflavin. A Simple Synthesis of the Substrate and Product of the Pyrimidine Deaminase and of Structural Analogs

P. Nielsen and A. Bacher

Lehrstuhl für Organische Chemie und Biochemie der Technischen Universität München, Lichtenbergstraße 4, D-8046 Garching, FRG

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2-Amino-5-nitro-6-ribitylaminopyrimidinone (7) was phosphorylated with chlorophosphoric acid yielding an isomer mixture containing about 63% of the 5'-phosphate 7a together with other monophosphates and bisphosphates of 7. Preparative HPLC afforded pure 7a. Catalytic hydrogenation of 7a yields the labile substrate of pyrimidine deaminase, 2,5-diamino-6-ribitylaminopyrimidinone 5'-phosphate (2a). The product of the enzyme, 5-amino-6-ribitylaminopyrimidinone 5'-phosphate (4a), can be obtained from 5-nitro-6-ribitylaminopyrimidinone (8) by an analogous procedure. The 3'- and 4'-monophosphates of 2,5-diamino-6-ribitylaminopyrimidinone-4(1H,3H)-pyrimidinedione are not substrates for the deaminase. 5'-Phosphates of a variety of ribityl-substituted pyrimidines and pteridines could also be obtained by phosphorylation with chlorophosphoric acid without the use of protecting groups.

Introduction

The biosynthesis of riboflavin is summarized in Fig. 1 (for review see Refs. [1—3]). It is firmly established that the first biosynthetic step consists in the opening of the imidazole ring of GTP leading to the pyrimidine intermediate 2a [4—6]. In yeasts, the ribose side chain of the intermediate 2a is reduced yielding 2,5-diamino-6-ribitylaminopyrimidinone 4(3H)-pyrimidinedione 5'-phosphate (2a) [7—10]. Subsequently, the position 2 amino group of the pyrimidine ring is hydrolyzed yielding 5-amino-6-ribitylaminopyrimidinone-4(1H,3H)-pyrimidinedione 5'-phosphate

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**Fig. 1. Biosynthesis of riboflavin.**

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* Present address: Physiologisch-Chemisches Institut, Abteilung Medizinische Biochemie, Universitätsklinikum Eppendorf, Martinistrasse 52, D-2000 Hamburg 20, FRG.

* Reprint requests to Dr. A. Bacher.

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The sequence of deamination and side chain reduction appears to be reverse in *Escherichia coli* with 3a as intermediate [15]. Dephosphorylation of 4a yields 4 which is subsequently converted to 6,7-dimethyl-8-ribityllumazine (5) by condensation with 3,4-dihydroxy-2-butano 4-phosphate [16–19]. The dismutation of 5 yielding riboflavin (6) and 4 which is catalyzed by riboflavin synthase has been studied extensively by Plaut and his coworkers [20, 21].

The detailed investigation of the early biosynthetic steps has been hampered by the instability and poor availability of the respective intermediates. The phosphoric acid esters 2a and 4a have been prepared by a sequence of tritylation and acetylation, followed by removal of the trityl protecting group and phosphorylation with cyanoethylphosphate [22]. Due to the insufficient regioselectivity of the tritylation reaction, the final product of this reaction contains only about 80% of the desired 5'-phosphate together with other phosphates.

This paper describes a simple procedure for the synthesis of the pyrimidines 2a and 4a without the use of protecting groups. The regioselectivity is comparable to the more laborious protection group approach [22]. Pure 5'-isomer can be obtained by preparative HPLC. A wide variety of structural analogs was also obtained by this method. A preliminary report of part of this work has been published [23].

### Experimental

#### General methods

Visible and UV spectra were obtained with a photometer PM 6 (Zeiss). The following extinction coefficients were used (pH 1.0): $7a, \epsilon_{334} = 14200 \text{M}^{-1} \text{cm}^{-1}$; $8a, \epsilon_{323} = 14200 \text{M}^{-1} \text{cm}^{-1}$. High performance liquid chromatography (HPLC) was performed with instruments from Waters Inc. and Hupe und Busch Co. Columns of Nucleosil 100–10 C18 (Macherey and Nagel, Düren; 4x250 mm, with a minimum of 4000 theoretical plates as determined with phenylacetic acid as solute and 30% methanol, adjusted to pH 3.0 by the addition of formic acid) were used for analytical work. Columns of Lichrosorb RP18 (Merck, Darmstadt; 16x250 mm) were used for preparative work. Phosphate was determined by published procedures [24]. Phosphoric acid esters were hydrolyzed by treatment with alkaline phosphatase (Boehringer, Mannheim) prior to the phosphate determination. Pyrimidine deaminase was partially purified from cell extract of *Candida guilliermondii* as published earlier [14].

#### Chemicals

The following compounds were prepared by published procedures: 6-chloro-5-nitro-2,4(1H,3H)-pyrimidinedione [25], 6,7-dimethyl-8-ribityllumazine [26], 5-nitroso-6-ribitylaminol-2,4(1H,3H)-pyrimidinedione [27], 2-amino-6,7-dimethyl-8-ribityl-4(3H)-pteridinone [28].

**5-Nitro-6-ribitylaminol-2,4(1H,3H)-pyrimidinedione (8) [25]**

A solution of 5-nitro-6-chloro-2,4(1H,3H)-pyrimidinedione (18 g, 94 mmol) in 360 ml of ethanol was added to a solution of 94 mmol ribitylamine [27] in 360 ml of water. The pH was repeatedly adjusted to 8 by the addition of 1 M K2HPO4. The mixture was heated at 60 °C for 1 h and then kept at room temperature overnight. The pH was adjusted to 10.7 by the addition of concentrated ammonia, and the precipitate formed was removed by filtration. The solution was evaporated to dryness. The residue was dissolved in 100 ml of water and placed on a column of Dowex 1X8 (100–200 mesh, formate form, 2.5x40 cm). The column was developed with (a) 1 l of water, (b) 1 l of 0.01 M formic acid, (c) 2 l of 0.1 M formic acid. Fractions containing 8 were pooled and concentrated to a small volume. The precipitate formed was collected. Yield, 5.37 g, 18.7%, m.p. 155 °C.

**Chlorophosphoric acid [29]**

Freshly distilled phosphorous oixitrichloride (7.3 ml, 80 mmol in a flask with stirrer) was immersed in an ice bath. Water (2.9 ml, 0.16 mol) was slowly added by a peristaltic pump over a period of 2–3 h. The product was used immediately.

**2-Amino-5-nitro-6-ribitylaminol-4(3H)-pyrimidinone 5'-phosphate (7a)**

A suspension of 7 (270 mg, 0.89 mmol) in 1.25 ml (16.5 mmol) of freshly prepared monochlorophosphoric acid was stirred for 26 h at room temperature. A stream of dry nitrogen was bubbled through the mixture to remove HCl. The reaction mixture was poured into 50 ml of dry ether. The solid was collected on a sintered glass filter by suction. The hygroscopic material was dissolved in 27 ml of water and brought to pH 4 by the addition of aqueous NH4OH.

The solution was applied to a column of DEAE cellulose DE 52 (25x17 cm, acetate form, equili-
brated with 30% 2-propanol). The column was developed with 50 ml of 30% 2-propanol and subsequently with a linear gradient of 0–0.5 M triethylammonium acetate pH 7 in 30% 2-propanol (total volume, 1.4 l). Fractions of 12 ml were collected. Starting material eluted in fraction 5–16 was recovered. A mixture of monophosphates was eluted in fractions 20–49. The fractions were combined and brought to dryness under reduced pressure. Triethylammonium acetate was removed by subsequent lyophilization. Yield, 276 µmol, 31% (determined photometrically). The mixture contained 63% 5'-phosphate, 25% 4'-phosphate and 11% 3'-phosphate as shown by HPLC.

Preparative HPLC

A solution of the isomeric phosphate mixture of 7 (35 µmol) in 5 ml of eluent (10 mM formic acid/10 mM ammonium formate) was applied in portions to a preparative HPLC-column (Lichrosorb RP18, 16×250 mm). The effluent was monitored at 335 nm and fractions were collected and lyophilized. Yield: 1.8 µmol 3'-phosphate (7c, 97% pure), 5.1 µmol 4'-phosphate (7b, 98% pure), and 16.9 µmol 5'-phosphate (7a, 97% pure).

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

A suspension of palladium on charcoal (1 mg) in 2 ml of water was saturated with hydrogen at room temperature and atmospheric pressure. 7a (12 mg) was added, and the mixture was stirred under an atmosphere of hydrogen overnight. The catalyst was removed by filtration through a nitrocellulose filter. The solution was used immediately.

5-Amino-6-ribitylamino-2,4(1 H,3 H)-pyrimidinedione 5'-phosphate (4a) was prepared from 8a as described for 2a.

Results and Discussion

We have studied the direct phosphorylation of 2-amino-5-nitro-6-ribitylamino-4(3H)-pyrimidinone (7, Scheme I) with chlorophosphoric acid. This method has been used extensively with riboflavin and a variety of riboflavin derivatives where the yield of desired 5'-phosphate is in the range of 70% of total product [29, 30]. The crude product obtained by phosphorylation of 7 contained starting material (A), several monophosphates (B–D) and small quantities of bisphosphates (Fig. 2, top left). The main product, B, appeared identical with the compound obtained earlier using the trityl derivative strategy. Ion exchange chromatography on DEAE cellulose (Fig. 2, top right) separated the monophosphates from the other components. Apparently pure monophosphates could be obtained by preparative HPLC on a reversed phase column using ammonium formate at pH 3 as eluent (Fig. 2, bottom). Amounts of about 1 mg can be run on a column of 16×250 mm. Each of the compounds B–D showed phosphate contents of 0.80–0.95 mol per mol of pyrimidine as detected after hydrolysis with alkaline phosphatase.

In an earlier study, we have analyzed the acid-catalyzed isomerization of riboflavin 5'-phosphate in considerable detail. These kinetic data can be summarized as follows:

\[
5'\text{-FMN} \xrightarrow{k_1} 4'\text{-FMN} \xrightarrow{k_2} 3'\text{-FMN} \xrightarrow{k_3} 2'\text{-FMN}
\]

All kinetic constants were in a narrow range of 1.5–9.5·10^-6 sec^{-1}. 5'-FMN had the highest thermodynamic stability, and the 5'-isomer is formed predominantly by treatment of unprotected riboflavin with chlorophosphoric acid [30]. We have utilized this experimental approach to characterize the pyrimidine phosphates B–D in closer detail. HPLC-purified compounds were treated with 0.1 M HCl at 50 °C, and the reaction mixtures were analyzed by HPLC at intervals (Fig. 3). Isomerization of compound B yields C in a relatively rapid first order reaction and D in a much slower reaction with sigmoidal kinetics. Isomerization of C yields B and D.
Fig. 2. HPLC chromatography of products obtained by phosphorylation of 2-amino-5-nitro-6-ribitylamino-4(3H)-pyrimidinone (7) with chlorophosphoric acid. Top left, crude product; top right, monophosphate mixture after chromatography on DEAE cellulose; bottom, 5'-phosphate after preparative HPLC. Column, Nucleosil RP$_8$, 4×250 mm; eluent, 10 mM ammonium formate plus 10 mM formic acid; detection, $\varepsilon_{280}$.

<table>
<thead>
<tr>
<th>Compound type</th>
<th>$k_1$</th>
<th>$k_{-1}$</th>
<th>$k_2$</th>
<th>$k_{-2}$</th>
<th>$k_3$</th>
<th>$k_{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 Phosphate</td>
<td>2.00</td>
<td>9.00</td>
<td>1.37</td>
<td>1.70</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Riboflavin phosphate$^a$</td>
<td>1.60</td>
<td>9.41</td>
<td>2.21</td>
<td>3.06</td>
<td>2.92</td>
<td>1.55</td>
</tr>
</tbody>
</table>

Table I. Velocities of acid catalyzed isomerization of the monophosphates of 2-amino-5-nitro-6-ribitylamino-4(3H)-pyrimidinone (7) and of riboflavin in 0.1 M HCl at 50 °C.

$^a$ Data from Nielsen et al. [30]; $^b$ ND, not determined.
The available evidence can be summarized as follows:

\[ B(7a) \xrightarrow{\text{k}_1} C(7b) \xrightarrow{\text{k}_3} D(7c) \]

The compounds \(7a\)–\(7c\) are formed at an approximate ratio of 5:2:1 by the reaction of \(7\) with chlorophosphoric acid. Thus, the regiospecificity is similar to that observed in the phosphorylation of unprotected riboflavin.

Catalytic hydrogenation of \(7a\) yields the labile \(2a\), the substrate of pyrimidine deaminase from \textit{Candida guilliermondii} [13, 14]. Treatment of this labile compound with diacetyl yielded the phosphorylated pteridine derivative \(14a\). In order to examine the substrate specificity of pyrimidine deaminase from \textit{C. guilliermondii}, the 5-nitropyrimidine \(7\) derivatives \(7a\)–\(7c\) were converted to the respective 5-amino compounds (\(2a\)–\(2c\)) by catalytic hydrogenation and incubated with partially purified pyrimidine deaminase as described earlier [14]. Only the 5'-phosphate was deaminated by the enzyme as shown in Table II. The enzymatic deamination of \(2a\) was not inhibited by the isomer \(2b\).

The product \(4a\) of the pyrimidine deaminase was obtained from \(8\) or \(9\) by phosphorylation and hydrogenation in analogy to the preparation of \(2a\). Reaction of \(2a\) with diacetyl yielded the lumazine derivative \(5a\). Phosphorylation with chlorophosphoric acid was also successful with a variety of substrate and product analogues of pyrimidine deaminase (Table III). The isomeric phosphoric acid esters could be separated by HPLC, but no detailed investigation has been performed. HPLC data are shown in Table III.
Table II. Substrate specificity of 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5'-phosphate deaminase (DRPP-deaminase). Enzyme assays were performed as described earlier [14]. Assay mixtures contained pyrimidine substrate at the concentration indicated which had been freshly prepared by catalytic hydrogenation of the respective precursor, 0.1 M phosphate buffer pH 7.0, 5 mM MgCl₂, 10 mM dithioerythritol, and partially purified pyrimidine deaminase from C. guilliermondii in a total volume of 0.5 ml. Assay mixtures were incubated at 37 °C for 1 h, and 100 μl of a solution containing 8 μmol diacetyl, 200 U of riboflavin synthase from Bacillus subtilis and 0.2 U of alkaline phosphatase were added. The mixtures were again incubated at 37 °C for 3 h, and riboflavin was monitored by bioassay with Lactobacillus casei.

<table>
<thead>
<tr>
<th>Substrate Riboflavin formed (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
</tr>
<tr>
<td>2a (0.63 mM)</td>
</tr>
<tr>
<td>2b (0.58 mM)</td>
</tr>
<tr>
<td>2c (0.10 mM)</td>
</tr>
<tr>
<td>2a+2b (0.31 mM)</td>
</tr>
<tr>
<td>2d (1.0 mM)</td>
</tr>
</tbody>
</table>

* 2 = 2,5-Diamino-6-ribitylamino-4(3H)-pyrimidinone.

Table III. Phosphorylation of 6-alditylaminoopyrimidines and pteridines.

<table>
<thead>
<tr>
<th>Product</th>
<th>Yield (%)</th>
<th>HPLC retention time (min)</th>
<th>Phosphate content (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>31.1</td>
<td>28.6</td>
<td>0.90</td>
</tr>
<tr>
<td>8a</td>
<td>23.5</td>
<td>13.0</td>
<td>1.05</td>
</tr>
<tr>
<td>9a</td>
<td>24.8</td>
<td>9.9</td>
<td>0.85</td>
</tr>
<tr>
<td>10a</td>
<td>21.9</td>
<td>9.8</td>
<td>0.90</td>
</tr>
<tr>
<td>11a</td>
<td>43.8</td>
<td>8.9</td>
<td>1.15</td>
</tr>
<tr>
<td>12a</td>
<td>35.3</td>
<td>12.6</td>
<td>0.95</td>
</tr>
<tr>
<td>13a</td>
<td>37.0</td>
<td>11.2</td>
<td>0.80</td>
</tr>
<tr>
<td>5a</td>
<td>7.0</td>
<td>36.5</td>
<td>0.92</td>
</tr>
<tr>
<td>14a</td>
<td>14.9</td>
<td>35.6</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* a Column, Nucleosil RP18, 4×250 mm; eluent: 10 mM ammonium formate/10 mM formic acid pH 3.7; flow rate, 0.7 ml/min; b determined after hydrolysis with alkaline phosphatase.

The reaction of the 5,6-diaminopyrimidine type compounds 2 and 4 with diacetyl yields the pteridine derivatives 5a and 14a [22]. These compounds can also be prepared by direct phosphorylation of the corresponding pteridines 5 and 14 with chlorophosphoric acid. However, the yield of the direct phosphorylation reaction was relatively low.

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