Isolation of 4-(1'-D-Ribitylamino)-5-amino-2,6-dihydroxypyrimidine from a Riboflavin-Adenine-deficient Mutant of Bacillus subtilis*

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Studies were carried out to determine possible intermediates involved in the biosynthetic pathway of riboflavin, using resting cells of a riboflavin-adenine-deficient mutant, Bacillus subtilis AJ1988. The cells excreted 6,7-dimethyl-8-ribityllumazine, the end product in the biosynthetic pathway, into the incubation medium in large amounts.

The addition of glyoxal caused a large accumulation of a green fluorescent compound; an inverse relation was observed between the formation of the lumazine and the concentration of glyoxal. Furthermore, added [2-14C]guanine effectively incorporated into the lumazine and the fluorescent compound in the same specific activity during incubation. The fluorescent compound was isolated, purified, and identified by paper chromatographic, fluorometric, and spectrophotometric analyses. It was proved to be 8-(1'-d-ribityl)lumazine, which appeared to have been formed by a reaction between glyoxal and a possible intermediate in the cells.

Accordingly, 4-(1'-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine was concluded to be an immediate precursor of 6,7-dimethyl-8-ribityllumazine.

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Experimental Procedures

Organism—A riboflavin-adenine-deficient mutant, Bacillus subtilis AJ1988, was the gift of Ajinomoto Co. Ltd. The cells were maintained at 28° on an agar slant and subcultured every 7 days.

Media—The agar slant contained 1% polypeptone, 1% yeast extract, 0.5% NaCl, and 1.5% agar, pH 7.0. The preculture medium consisted of the nutrients used in the agar slant without the agar. The basal medium contained 6% glucose, 1.5% NH₄Cl, 0.1% KH₂PO₄, 0.04% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 0.001% MnSO₄·7H₂O, 0.048% (as total nitrogen) soy bean hydrolysate, 0.04% adenosine, and 1 μg/100 ml riboflavin, pH 7.0. The resting cell medium (a minimal medium) contained 1% glucose, 0.1% (NH₄)₂SO₄, 0.05% sodium citrate, 0.02% MgSO₄·7H₂O, 0.846% KH₂PO₄, and 0.23% KOH, pH 7.0.

Chemicals—Glyoxal (40% in water) was obtained from Nakarai Chemicals, Ltd., Kyoto, Japan. [2-14C]Guanine was obtained from Commissariat à l'Energie Atomique, Gif-sur-Yvette, France, through Japan Radio Isotope Association, Tokyo, Japan. 6,7-Dimethyl-8-(1'-d-ribityl)lumazine and 8-(1'-d-ribityl)lumazine were synthesized by the methods of Plaut and Harvey (26) and Masuda (27). Acid alumina for column chromatography was obtained from M. Woelm Co., Eschwege, Germany. Dowex 50W-X4 and -X8 (200 to 400 mesh), and Dowex I-X2 (200 to 400 mesh) were obtained from Dow Chemical Co., Midland, Mich.; DEAE-cellulose from Serva AG, Heidelberg, Germany; and Sephadex G-10 from Pharmacia Fine Chemicals, Uppsala, Sweden.

Paper Chromatography—The following solvent systems were used: A, 1-butanol:acetic acid:water (30:20:20, v/v); B, 1-butanol: ethanol:water (50:15:35, v/v); C, 1-butanol:pyridine:water (60:40:30, v/v); D, tert-butyl alcohol:water (60:40, v/v); E, tert-butyl alcohol: NH₄OH (concentrated):water (60:3:35, v/v); F, tert-butyl alcohol:pyr...
Isolated compound
R-Ribityllumazine
solution with the crystals was paper chromatographed.

nucleotide monophosphate is based on the results of Boris Magasa-

The clear fluid obtained was used to determine the amounts of 6,7-

The green fluorescent compound was isolated from the resting cell medium containing 0.03% glyoxal which was incubated at 31°C for 20 h.

Isolation and Purification of Accumulated Compound—After resting cell incubation, the medium (8340 ml) without cells was evaporated below 30°C under reduced pressure, to about 800 ml and then the concentrated fluid was centrifuged. The supernatant (60 ml) was applied to a column (3.2 x 30 cm) of Dowex 50W-X4-H+ and eluted with distilled water at a flow rate of 40 ml/min. The green fluorescent fractions were collected under ultraviolet light. This purification procedure was repeated. Dowex 1-X2-OH resin was added to the solution (5 liters total volume) to adjust the pH to 5.0 and the suspension was filtered through paper. The resin was washed with distilled water, and the washings were added to the clear filtrate obtained above. The combined solution (7.5 liters) was concentrated to 400 ml under reduced pressure. One hundred milliliters of the solution was loaded on a column (3.2 x 30 cm) of DEAE-cellulose (OH-), then washed with distilled water, after which it was eluted with 5 mM acetic acid at an initial flow rate of 20 ml/min. This process was repeated four times after which the combined fluorescent eluate was evaporated to dryness. The residue was dissolved in distilled water (20 ml) and applied to a double layer column (3.2 x 30 cm) which had first been packed with Dowex 50W-X8-H+ to a height of 30 cm then with Dowex 1-X2-C1 to a height of 55 cm. The column was eluted with distilled water at a flow rate of 40 ml/min. The green fluorescent fractions were combined and directly run through a column (3.2 x 30 cm) of DEAE-cellulose (OH-), followed by washing and elution with the above solution. Then these fractions were collected and dried in vacuo, after which they were dissolved in 10 ml of distilled water. Forty milliliters of ethanol were added to the solution and the mixture was applied to a column (2.2 x 25 cm) of acid alumina which had been washed with absolute ethanol until the ultraviolet absorbance of the washings was negligible. The column was then washed with 80% ethanol, and elution was carried out with 50% ethanol at an elution rate of 20 ml/5 min after which the eluate was evaporated to dryness. The dried material was dissolved in a minimum volume (3 ml) of distilled water, then it was placed on a column (1.5 x 80 cm) of Sephadex G-10 and eluted with distilled water at a flow rate of 2.5 ml/10 min. The fluorescent fluid obtained was evaporated to a small volume (5 ml). Absolute ethanol at room temperature was added to the resulting solution to give an 80% ethanol solution which was left to crystallize in the cold. Recrystallization was done up to 3%.

The yield of crystals was 4 mg from the resting cell medium (8340 ml). The crystals were pale yellow and emitted a brilliant green fluorescence in an aqueous solution under ultraviolet light.

Tracer Experiments with Labeled Guanine—Resting cell incubation was done in the resting cell medium (20 ml) in a 500-ml Erlenmeyen flask containing 0.05% glyoxal and 6.47 x 10-6 cm2 of 12-C guanine at 10-4 M, by the method described above. After incubation the suspension was centrifuged. The cells (0.36 g) were suspended in 2.5 ml of distilled water and heated on a water bath at 80°C for 10 min. The slurry was centrifuged. The supernatant (1.25 ml) was combined to the medium (10 ml) obtained above, which was applied to a double layer column of Dowex 50W-X4-H+ and Dowex 1-X2-(HCOO-) (0.9 x (14 + 4) cm). The column was eluted with distilled water at a flow rate of 3 ml/min. The 8-ribityllumazine fraction or the 6,7-dimethyl-8-ribityllumazine fraction was separately placed on a column (0.9 x 15 cm) of DEAE-cellulose (OH-), then eluted with 8 mM acetic acid at an initial flow rate of 1 ml/min. The eluate was collected in batches of 3 ml and used to determine the absorbance at 398 nm for 8-ribityllumazine or at 408 nm for 6,7-dimethyl-8-ribityllumazine and the radioactivity.

For determination of radioactivity, 0.5 ml of each fraction was dried and its radioactivity was counted in a toluene scintillant (16). The contents of 6,7-dimethyl-8-ribityllumazine and 8-ribityllumazine were determined from their absorbances using the extinction coefficients of 10.2 mm'cm-' at 408 nm for 6,7-dimethyl-8-ribityllumazine and of 9.5 mm'-cm-' at 398 nm for 8-ribityllumazine (28).

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
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<tbody>
<tr>
<td>8-Ribityllumazine</td>
<td>0.37</td>
<td>0.24</td>
<td>0.27</td>
<td>0.40</td>
<td>0.50</td>
<td>0.56</td>
<td>0.30</td>
<td>0.63</td>
<td>0.33</td>
<td>0.46</td>
</tr>
<tr>
<td>Isolated compound</td>
<td>0.27</td>
<td>0.25</td>
<td>0.27</td>
<td>0.40</td>
<td>0.50</td>
<td>0.56</td>
<td>0.30</td>
<td>0.63</td>
<td>0.33</td>
<td>0.46</td>
</tr>
</tbody>
</table>

**Fig. 1.** Positions of the mutation in a riboflavin-adenine-deficient mutant, *Bacillus subtilis* A388. The interconversion pathway of the riboflavin mononucleotide is based on the results of Boris Magasnik (41). AICAR, 5-amino-4-imidazolecarboxamide ribotide; DMRL, 6,7-dimethyl-8-ribityllumazine.
Specific activities are expressed as counts per min per μmol. In the calculation of the specific activity of 6,7-dimethyl-8-ribityllumazine, the values which were obtained by subtracting the amounts of 6,7-dimethyl-8-ribityllumazine at the start of incubation from those at the 20th h, were used as the true amounts of the lumazine formed during incubation.

RESULTS

Identification of Unknown Green Fluorescent Compound

Accumulated in Presence of Glyoxal—Paper chromatographic comparisons of the isolated compound and the synthesized 8-((1'-D-ribityl)lumazine were made with various organic solvents. Table I shows that the Rf values of the isolated compound are similar to those of 8-ribityllumazine. But, these values clearly differ from those of 6,7-dimethyl-8-ribityllumazine which has larger ones in the solvents used (29). The crude extract obtained by heating an aqueous cell suspension and the incubation fluid showed no riboflavin on the paper chromatograms developed in these solvents.

The ultraviolet and visible absorption spectra of the isolated compound were examined over a wavelength range of 210 to 470 nm in 0.05 M phosphate buffer (pH 7.0), 0.1 N HCl, and 0.1 N NaOH, and were compared to those of synthesized 8-ribityllumazine and 6,7-dimethyl-8-ribityllumazine. Table II shows that their absorption maxima are quite similar in the acid and alkaline solutions. However, these compounds clearly differ from pterins and lumazines with no substituent at nitrogen 8; these compounds have an absorption maxima near 330 nm in the acid and neutral solutions (13, 14, 20). Thus, these results mean that the pteridine ring of the isolated compound contains a substituent on nitrogen 8.

Furthermore, the excitation spectra of these compounds were studied in 0.05 M phosphate buffer (pH 7.0); the fluorescence intensity at 500 nm was determined over a range of excitation wavelengths from 210 to 500 nm. The emission spectra of these compounds were determined by recording the fluorescence intensity over a wavelength range of 400 to 600 nm using an excitation wavelength of 398 nm. The results of the excitation spectra experiment agreed well with those of the ultraviolet absorption spectra of these compounds in the same buffer, especially in the absorption maxima at 398 nm. The maximum fluorescence intensity at 480 nm in these emission spectra also indicated a characteristic of ribityllumazine type compounds (21, 29, 30) which differ from pterins and lumazines having no side chain at nitrogen 8; the latter show a maximum fluorescence intensity near 455 nm and 440 nm at the pH used (20).

The infrared spectra of the isolated compound and the synthesized 8-ribityllumazine were examined using a KBr pellet method. Fig. 2 shows that the spectrum of the isolated compound was identical with that of the synthesized 8-ribityllumazine. The spectrum of the isolated compound differed little from those for 6,7-dimethyl-8-ribityllumazine (29) and 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine (12).

Demonstration of the identity of the structures of the isolated compound and the synthesized 8-ribityllumazine was made by mass spectrometry. This was performed by the direct inlet method. Results are shown in Fig. 3. The spectra did not show a molecular ion. However, a peak at m/e 178 indicates 8-methylumazine obtained through partial degradation of the side chain attached to nitrogen 8 on the molecule of the isolated compound. The fragment at m/e 164 represents lumazine, which is formed from the complete destruction of the side chain. Furthermore, ions at m/e 86 and 103 correspond to fragments derived from the side chain, itself, of the isolated compound, 3,4-dihydroxytetrahydrofuran and 3-hydroxy-2,3-dihydrofuran. These fragments were likewise detected in the patterns of 6,7-dimethyl-8-ribityllumazine and 2-amino-6,7-dimethyl-4-oxo-8-ribitylpteridine. The base ion was observed at m/e 57. In addition, Fig. 3 shows that the fragmentation pattern of synthetic 8-ribityllumazine is the same as that of the isolated compound although there are small differences in the intensity of the respective fragments in the two spectra. The fragmentation pattern shown in Fig. 4 results from a comparison with those of similar pterins and lumazines (12, 31).

Accordingly, results of all the ultraviolet, excitation, emission, infrared, and mass spectra and of comparative paper chromatography demonstrate that the isolated compound is 8-((1'-D-ribityl)lumazine. This was also ascertained by measuring the optical rotatory dispersion of these compounds. Although no clear patterns were obtained because of interfer-
ence, due to their high absorptions in the regions of the wavelength used (240 to 600 nm), the behavior of the isolated compound almost completely coincided with that of the synthesized 8-(1'-n-ribityl)lumazine (c = 0.04).

Relation of 8-Ribityllumazine Accumulated to 6,7-Dimethyl-8-Ribityllumazine Produced in Presence of Glyoxal—The effect of glyoxal at various concentrations on the production of 6,7-dimethyl-8-ribityllumazine was examined in the resting cell medium with a riboflavin-adenine deficient mutant of Bacillus subtilis. Addition of glyoxal to the resting cell medium caused a sharp inhibition of 6,7-dimethyl-8-ribityllumazine formation and simultaneously a marked accumulation of 8-ribityllumazine, showing an inverse relation over a concentration range of 0 to 0.03% glyoxal under the conditions used. Above this range, both curves decreased with increasing concentrations of the reagent. The yields of 6,7-dimethyl-8-ribityllumazine amounted to 2720 µg/g of wet cells in the absence of glyoxal, and those of 6,7-dimethyl-8-ribityllumazine and 8-ribityllumazine were 762 and 508 µg/g of wet cells, respectively, in the presence of 0.63% glyoxal.

In resting cells of the mutant fed with glyoxal and [2-14C]guanine, we sought evidence that the accumulated 8-ribityllumazine is an intermediate derivative in the riboflavin biosynthetic pathway. Results are shown in Table IV. A comparison of the specific activities of both lumazines with

**Table IV**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific Activity</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Ribityllumazine</td>
<td>1.30 x 10^6</td>
<td>1.08</td>
</tr>
<tr>
<td>6,7-Dimethyl-8-ribityllumazine</td>
<td>1.20 x 10^6</td>
<td>1.00</td>
</tr>
</tbody>
</table>

FIG. 3. Mass spectra of the isolated compound and 8-(1'-n-ribityl)lumazine.

FIG. 4. Proposed fragmentation pattern of the isolated compound and 8-(1'-n-ribityl)lumazine.
that of the guanine initially added indicates that guanine is effectively incorporated into 6,7-dimethyl-8-ribityllumazine and 8-ribityllumazine with about 5-fold dilution, respectively. Moreover, the specific activity of 6,7-dimethyl-8-ribityllumazine coincided well with that of 8-ribityllumazine; the ratio of specific activities was 1.00:1.08.

**DISCUSSION**

Studies on possible intermediates involved in flavinogenesis were made with resting cells of a riboflavin-adenine-deficient mutant, *Bacillus subtilis* AJ 1988. Cells could not synthesize riboflavin, which was confirmed by paper chromatography with the cell extract and the incubation fluid. However, the immediate precursor of riboflavin, 6,7-dimethyl-8-ribityllumazine, was excreted in large quantities from the cells during cultivation on a basal medium and also during resting cell incubation. Therefore, the mutant cells appear to lack riboflavin synthetase (EC 2.5.1.9) which catalyzes the conversion of 6,7-dimethyl-8-ribityllumazine to riboflavin (23, 24).

When glyoxal (0.03%) was added to the resting cell medium with mutant cells, the accumulation of a green fluorescent compound was caused simultaneously with the inhibition of 6,7-dimethyl-8-ribityllumazine formation. The accumulation occurred at levels comparable to the lumazine produced. Thus, the unknown fluorescent compound, which clearly differed from 6,7-dimethyl-8-ribityllumazine, appears to have an intimate relation to flavinogenesis. This compound was isolated from the resting cell medium after 20 h of incubation in the presence of glyoxal (0.03%) and purified to a crystalline form through column chromatography. The purified compound was identified by a paper chromatographic comparison and by determination of the ultraviolet, excitation, emission, infrared, and mass spectra and by optical rotatory dispersion. The results clearly indicate that the purified compound is 8-[(1'-n-ribitylamino)-5-amino-2,6-dihydroxypyrimidine. Based on the assumption that glyoxal serves merely as a trapping agent, the compound which reacts with glyoxal in the cells appears to be 4-(1'-d-ribitylamino)-5-amino-2,6-dihydroxypyrimidine; this compound would be expected to react with glyoxal to form ribityllumazine. The structural similarity of 4-(1'-d-ribitylamino)-5-amino-2,6-dihydroxypyrimidine and 6,7-dimethyl-8-ribityllumazine indicates that the pyrimidine is the nearest intermediate to 6,7-dimethyl-8-ribityllumazine in the pathway of riboflavin biosynthesis. This interpretation was strengthened by the experiments with labeled guanine, which showed that added guanine actively incorporates into the 6,7-dimethyl-8-ribityllumazine and the accumulated 8-ribityllumazine to the same extent of dilution and thus the specific activities of both lumazines are identical.

The same pyrimidine was previously isolated from mutants of *Saccharomyces cerevisiae* (11) and *Pichia guilliermondii* (14). However, its structure and relation to flavinogenesis were not made clear in the reports although riboflavin-deficient mutants were used.

The pyrimidine has the same structure as the byproduct formed in the riboflavin synthetase reaction because the byproduct was identified as 4-(1'-ribitylamino)-5-amino-2,6-dihydroxypyrimidine through the entrapping method using glyoxal in the reaction medium with riboflavin synthetase (30). Furthermore, we verified that the byproduct is reutilized in vitro to form riboflavin in the presence of diacetyl (32). Therefore, it is quite reasonable that in normal cells the byproduct is utilized as the immediate precursor of 6,7-dimethyl-8-ribityllumazine right after the riboflavin synthetase reaction. However, the mutant cells can not produce the byproduct because of the lack of riboflavin synthetase; thus, the cyclization of the byproduct in such a way appears not to occur in the cells.

In addition, we showed that a direct nucleotide precursor is guanosine triphosphate (GTP) in experiments with labeled

![Diagram of the proposed pathway of riboflavin biosynthesis](https://via.placeholder.com/150)

**Fig. 5.** Proposed pathway of riboflavin biosynthesis. RAADP, 4-(1'-ribitylamino)-5-amino-2,6-dihydroxypyrimidine; MHRL, 6-methyl-7-hydroxy-8-ribityllumazine; DMRL, 6,7-dimethyl-8-ribityllumazine.
purines using a high flavinogenic mold, Erremothecum ashei-
byi (16, 33). Accordingly, GTP is believed to be converted to
4-ribitol(4-ribofuranosyl)aminopyrimidine-2,5-diamino-6-
hydroxypyrimidine, accompanied by the elimination of carbon 8 after
rupture of the imidazole ring (2, 5, 6).

Recently, Bacher and Lingens (12, 13) and Logvinenko et al.
(14) isolated two pyrimidines, 2,4,5-triamino-6-hydroxypy-
rimidine and 4-ribitylaminopyrimidine, which they considered to be immediate intermediates
after GTP, based on their structures. They indicated the possibility that the former is a derivative formed during
isolation or excretion through the cell membrane from the
riboflavin. Therefore, the structure of the immediate inter-
mediate after GTP remains obscure.

In the biosynthetic pathway of riboflavin, it is clear that
GTP is transformed to another pyrimidine, 4-(1'-d-ribityla-
mino)-5- amino-2,6-dihydroxypyrimidine through the equivo-
cal pyrimidine intermediates described above. The pyrimidine
intermediate, 4-ribitylaminopyrimidine-5-amino-2,6-
dihydroxypyrimidine, reacts with the "4-carbon compound" to form 6,7-di-
methyl-8-ribityllumazine. The 4-carbon compound obligatory
for the pathway has yet to be identified (7, 8, 35-37). Our
results, which were obtained in the reutilization reaction
(39) from 6,7-dimethyl-6-ribityllumazine and to inhibit the
riboflavin synthetase reaction at a low concentration (K, =
10^{-2} M) (40), is shown as an inhibitor of the biosynthetic
pathway of riboflavin.

More detailed evidence is needed to determine the origins of
the active 4-carbon compound, the ribityl side chain and the structure of the immediate intermediate after GTP in the
biosynthetic pathway of riboflavin.
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