Nous avons déterminé que les températures de transition entre les hydrates à 9 et 10 H_2O et entre les hydrates à 8 et 9 H_2O sont 40.0° et 54.3°. Ces températures de transition coïncident bien avec les valeurs obtenues par la mesure de la pression de la vapeur d'eau de dissociation[1] des hydrates de DHC.

La solubilité du DHC dans l'eau augmente avec la température dans la courbe de solubilité (Fig. 1), mais pour la courbe de viscosité-température, on remarque que la viscosité diminue avec l'augmentation de la température. Nous considérons que la cause de ce fait est la suivante : pour indiquer la viscosité nous avons pris le temps de chute de la solution dans le tube capillaire ; l'augmentation de la solubilité est relativement petite à des températures comprises entre 20° et 60°, et l'influence de la diminution de viscosité sur l'augmentation de la température du solvant est supérieure à l'influence de l'augmentation de viscosité sur l'augmentation du corps dissous.

Résumé

Nous avons obtenu la courbe de solubilité et la courbe de viscosité-température, en mesurant la solubilité du diphénylhydantoïnate de calcium dans l'eau et la viscosité de la solution saturée à des température comprises entre 20° et 60°, et nous avons déterminé les températures de transition des hydrates du diphénylhydantoïnate de calcium d'après le fait que les points de discontinuité dans ces courbes correspondent aux points de transition des parties solides (hydrates de DHC) qui coexistent avec la solution saturée.

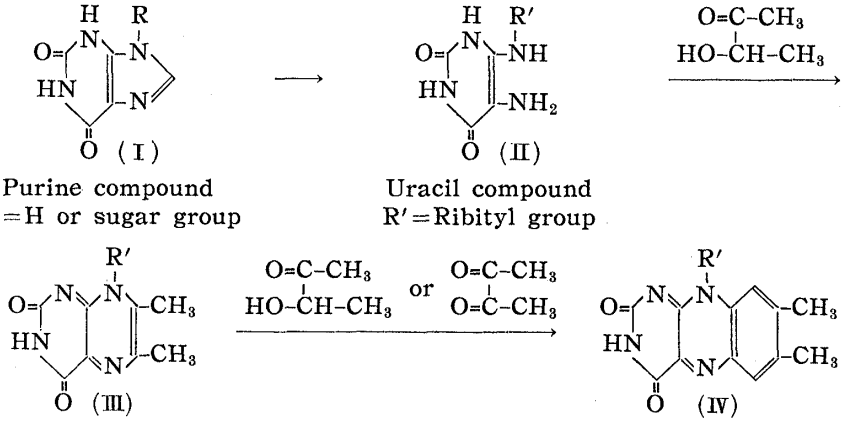
(Reçu le 20 Août, 1958)

UDC 547.859 : 545.84

70. Toru Masuda, Toyokazu Kishi, Mitsuko Asai, and Satoru Kuwada : Application of Chromatography. XXXVII.*1 Total Synthesis of 6,7-Dimethylribolumazine.

(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.**2)

Masuda,[1] one of the authors, isolated a green fluorescent substance, 6,7-dimethylribolumazine, from the mycelium of *Eremothecium ashbyii* and established it as an intermediate in the biosynthesis of riboflavin.



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*1 Part XXXVI : This Bulletin, **6**, 618(1958).

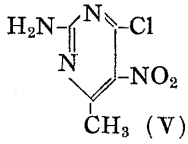
*2 Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (増田 亨, 貴志豊和, 浅井満子, 桑田 智).

1) T. Masuda : This Bulletin, **4**, 375(1956); **5**, 136(1957).

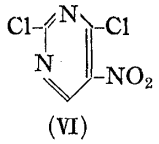
The present authors undertook the synthesis of this biochemically significant compound to ascertain its structure and first attempted to prove whether the route for the biosynthesis of riboflavin described in Part XXXII[2] of this series could be effected even *in vitro*.

Of the compounds of this series, the uracil compound with a ribityl group (II) provoked the authors' interest for its synthesis, because it has not been isolated from natural products nor synthesized up to now.

In the first place, attempt was made to obtain 4-ribitylaminouracil (IX) by the reaction of 4-chlorouracil (VII), which was prepared by Masuda[3] as an intermediate in the synthesis of 6,7,8-trimethyllumazine, with D-ribamine[4,5] at 120° in an autoclave, but all attempts, including chromatography, for crystallizing the product were unsuccessful. Todd, *et al.*[6] once reported that they carried out reactions between 1-aminoglucose and various 4-halopyrimidines under various conditions, but even the compounds (V) and (VI), the chlorine of which at 4-position is strongly activated by the nitro group at 5-position, did not give satisfactory results.



(V)



(VI)

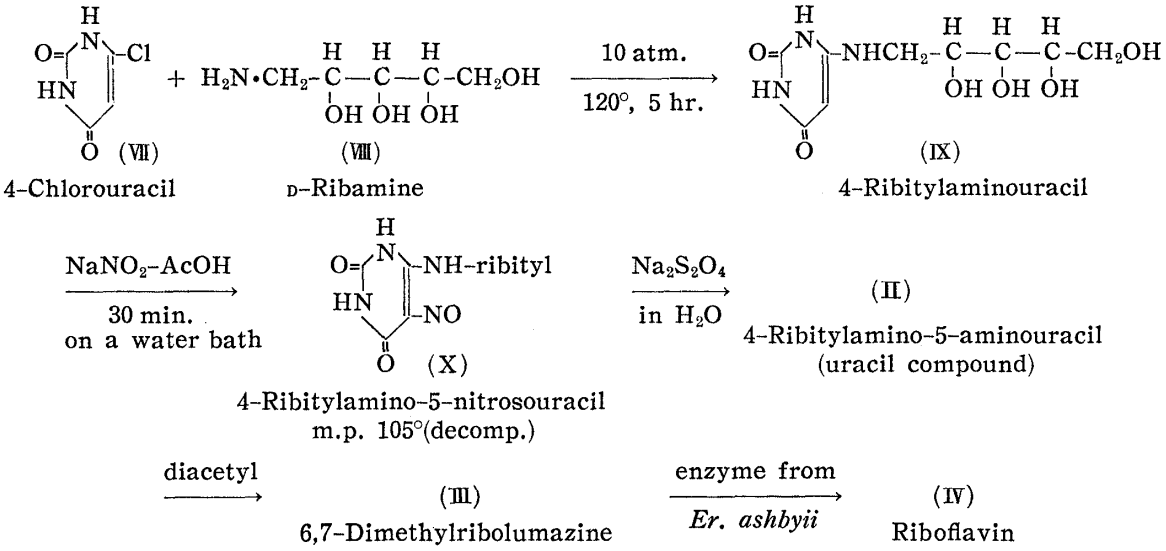
Even from this report alone, the above-mentioned reaction does not seem to proceed smoothly under such conditions, but formation of a ribityl derivative was presumed from the fact that the sparingly soluble starting material became soluble.

The above condensation mixture was concentrated and, after being acidified with acetic acid, allowed to react with sodium nitrite. The reaction mixture was poured on a column of activated charcoal, eluted with a diluted aqueous solution of pyridine, and the fractions, which were decolorized by sodium hydrosulfite ($Na_2S_2O_4$) and which gave yellow color with Ehrlich reagent, were collected. The combined fraction was again subjected to the same purification procedure and the product was isolated as orange-red rods, m.p. 105°. The analytical values were in complete accord with 4-ribitylamino-5-nitrosouracil (X) plus one mole of water of crystallization, $C_9H_{14}O_7N_4 \cdot H_2O$.

To produce the desired uracil compound (II) by reduction of the nitroso compound, sodium hydrosulfite was selected as a mild reducing agent. As mentioned before, a solution of the nitroso compound was rapidly decolorized by addition of the reducing agent, and the reaction mixture gave a spot positive to Ehrlich reaction on filter paper at Rf 0.25, 0.35, and 0.00 when developed with EtOH•BuOH•H_2O (15:50:35), pyridine•BuOH•H_2O (3:4:7), and AcOH•BuOH•H_2O (1:4:5), respectively, showing the formation of an amino compound. Therefore, the paper chromatography was repeated using a large sheet of filter paper and developing with EtOH•BuOH•H_2O, and the spot corresponding to Rf 0.25 was extracted with water. The extract was concentrated, treated with alcohol, and crystals were separated from the resulting viscous substance. However, the product as well as its mother liquor exhibited an extremely faint Ehrlich reaction. Judging from this fact, it seems that the 4-ribitylamino-5-aminouracil (II) once produced by reduction of the nitroso compound changed during the procedure and lost its amino nature.

2) T. Masuda : *Ibid.*, **6**, 291(1958). See also Footnote (1).
3) T. Masuda : *Ibid.*, **5**, 28(1957).
4) R. Kuhn, K. Reinemund, F. Weygand, R. Ströbele : Ber., **68**, 1765(1935).
5) R. Kuhn, P. Desnuelle, F. Weygand : *Ibid.*, **70**, 1293(1937).
6) J. Baddiley, B. Lythgoe, A. R. Todd : J. Chem. Soc., **1943**, 571.

Next, the reduction mixture was immediately reacted with diacetyl. The reaction mixture was acidified with acetic acid, poured on a Florisil column, and, after washing successively with diluted acetic acid and water, eluted with 3% aqueous solution of pyridine. From the green fluorescent fraction of the eluate, crystals of m.p. 272~274° (decomp.) were separated. This product was in complete agreement with the 6,7-dimethylribolumazine isolated from the mycelium of *Er. ashbyii*. Moreover, it was proved that this product yields riboflavin by action of a crude enzyme solution prepared from *Er. ashbyii*.



Thus, the total synthesis of 6,7-dimethylribolumazine was completed by the present work and its structure was thereby established. As mentioned before, the uracil compound, i.e. 4-ribitylamino-5-aminouracil, though not treated under drastic conditions, could not be obtained pure. It is not yet clear why this compound cannot be detected in the mycelium of *Er. ashbyii* but it may be that the compound is not produced originally or it combines with acetoin or with pyruvic acid immediately after its formation, or it is lost during the procedure because of its minute quantity.

In a recent paper, Plaut, *et al.*[7] briefly stated that they synthetically evidenced a green fluorescent substance isolated from *Ashbya gossypii* to be 6,7-dimethyl-8-ribityllumazine. The authors, however, have not yet had the chance to hear from them about the details of the work.

The authors are grateful to Messrs. Masao Nishikawa and Hiroshi Okuto for the physicochemical measurements and to Mr. Ichiro Uchida for his assistance in high-pressure experiments. Thanks are also due to members in charge of elementary analysis.

Experimental

D-Ribose Oxime—According to the method of Kuhn, *et al.*,[4] a hot EtONa-EtOH solution, prepared from 1.4 g. of metallic sodium and 35 cc. of dehyd. EtOH, was added to a hot solution of 4 g. of $NH_2OH \cdot HCl$ in 40 cc. of dehyd. EtOH and the mixture was heated for 15 min. After cool, the mixture was filtered and to the clear filtrate, 6 g. of D-ribose was added in small portions at 60~70°, and the resulting crystals, m.p. 140°, were collected.

D-Ribamine (VIII)—Following the procedure of Kuhn, *et al.*,[5] 3 g. of D-ribose oxime was dissolved in 20 cc. of water with warming and the solution was shaken with a suspension of 1 g. of Pt-black in ca. 20 cc. of water in H_2 atmosphere, when a theoretical amount (814 cc.) of H_2 was absorbed in about 6 hr. The establishment of the resulting D-ribamine was made by the method of Kuhn, *et al.*[8] After filtering off the catalyst, the filtrate was concentrated, and a part of the remaining syrup

7) G. F. Maley, G. W. E. Plaut: Federation Proc., **17**, 268(1958).
8) R. Kuhn, G. Wendt: Ber., **81**, 553(1948).

was isolated as oxalate, m.p. 138°. The syrup was also heated with freshly distilled acetylacetone (b.p. 137°) and dehyd. EtOH, and the reaction mixture, after addition of AcOEt, was left standing over night. The supernatant was concentrated and the separated crystals were recrystallized from acetone to colorless thin plates, m.p. 119°. *Anal.* Calcd. for $C_{10}H_{19}O_5N$ (Acetylacetone derivative of D-ribamine): C, 51.50; H, 8.19; N, 6.00. Found: C, 51.53; H, 8.20; N, 6.05.

4-Ribitylaminouracil (IX)—An amount of 1.5 g. of 4-chlorouracil[3](VII) was added to a solution of crude D-ribamine prepared by the catalytic reduction of 3 g. of D-ribose oxime and the mixture was heated in an autoclave at 120° for 5 hr. at 10 atm. The reaction mixture was filtered and the brown filtrate was concentrated in order to separate crystals or purified by chromatography on activated charcoal, but all these attempts to obtain a single product were unsuccessful.

4-Ribitylamino-5-nitrosouracil (X)—The same reaction mixture as above was concentrated to 20 cc. and then heated with 3 cc. of AcOH and 2 g. of $NaNO_2$ for 30 min. on a water bath. The reaction mixture was poured on a column (2.5×20 cm.) packed with 20 g. activated charcoal and eluted with 2~5% aq. solution of pyridine, obtaining the following fractions:

1) 200 cc., colorless; 2) 50 cc., yellow; 3) 30 cc., light red; 4) 120 cc., rose-red; 5) 50 cc., orange-red.

Of the five fractions, 3 and 4 were decolorized by $Na_2S_2O_4$ solution and the decolorized solutions gave yellow color with Ehrlich reagent.

The combined fractions 3 and 4 were concentrated and several volumes of EtOH was added. After filtering off the separated brown impurities, the filtrate was evaporated to dryness, leaving 200 mg. of a red residue. The residue was dissolved in a little water, the solution was purified by chromatography on activated charcoal as above, and a part of the eluate showing rose-red color was concentrated and allowed to stand, whereupon red crystals separated out. The product was recrystallized from water to beautiful orange-red rods, m.p. 105°(decomp.). The yield, however, was as poor as 5% of the theoretical based on 4-chlorouracil. Analytical values (after drying at 80° for 3 hr.) were in complete agreement with the captioned nitroso compound plus one mole of water of crystallization. *Anal.* Calcd. for $C_9H_{14}O_7N_4 \cdot H_2O$: C, 35.07; H, 5.33; N, 18.18. Found: C, 34.99; H, 5.21; N, 18.48.

4-Ribitylamino-5-aminouracil (II)—To a solution of 10 mg. of the 5-nitroso compound in 1 cc. of water ca. 100 mg. of $Na_2S_2O_4$ was added, when the solution was decolorized. The reaction mixture gave yellow color with Ehrlich reagent and exhibited green fluorescence by addition of diacetyl, fully endorsing the formation of an amino compound. The whole reaction mixture was concentrated and developed on a filter paper (30×40 cm.), with EtOH·BuOH·H_2O (15:50:35), and the spot corresponding to Rf 0.25, which was positive to Ehrlich reaction, was extracted with water. EtOH was added to the extract and the separated viscous substance was left standing for a long time, whereupon crystals separated out. However, the product as well as its mother liquor exhibited a very faint Ehrlich reaction, showing that the once produced amino compound changed during the above procedure. Further study on this problem is now under way.

6,7-Dimethylribolumazine (III)—A solution of 10 mg. of the 5-nitroso compound in 3 cc. of water was reduced with 100 mg. of $Na_2S_2O_4$ and the reaction mixture was immediately heated with 50 mg. of diacetyl on a water bath for 30 min. After cool the reaction mixture was mixed with 5% its volume of AcOH, poured through a column (2×15 cm.) of 50 g. of Florisil, the column was washed successively with 100 cc. of 5% AcOH and 100 cc. of water, eluted with 3% aqueous solution of pyridine. The first ca. 100 cc. of the eluate was discarded and the next 150 cc., which exhibited strongly green fluorescence, was concentrated and several volumes of EtOH was added. The separated inorganic impurities were filtered off, the filtrate was further concentrated, and, after addition of five volumes of EtOH, allowed to stand in a cool place, whereupon light green needles separated out. Recrystallization from 80% EtOH afforded 7 mg. of crystals of the same form, m.p. 272~274° (decomp.), $[\alpha]_D^{20}$ −148°(c=0.5, H_2O); Rf 0.13 (AcOH·BuOH·H_2O (1:4:5)), 0.22 (EtOH·BuOH·H_2O (15:50:35)), 0.53 (pyridine·BuOH·H_2O (3:4:7)).

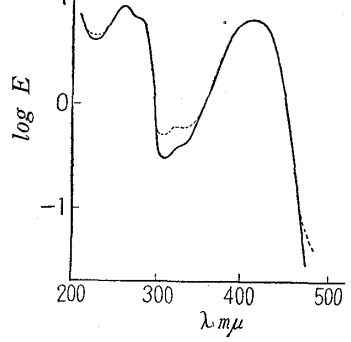


Fig. 1.
Ultraviolet Absorption Spectra of 6,7-Dimethylribolumazine from *Eremothecium ashbyii* and Synthesized Product

—— Synthesized 6,7-dimethylribolumazine
------ 6,7-Dimethylribolumazine from *Er. ashbyii*

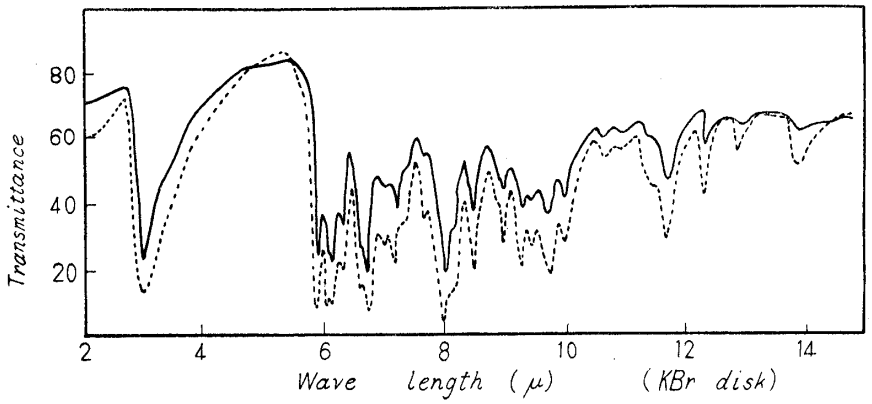
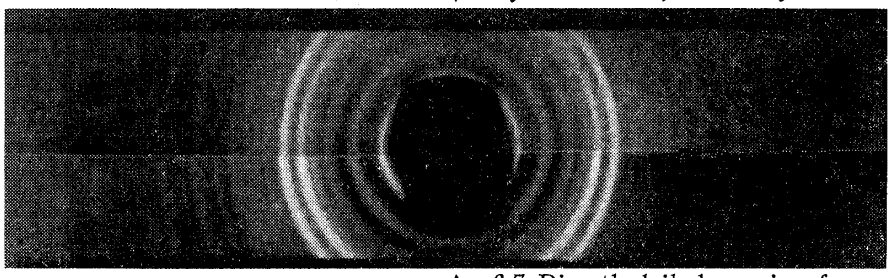


Fig. 2. Infrared Absorption Spectra of 6,7-Dimethylribolumazine from *Er. ashbyii* and Synthesized Product
—— Synthesized 6,7-dimethylribolumazine
------ 6,7-Dimethylribolumazine from *Er. ashbyii*

↓ Synthesized 6,7-dimethylribolumazine



↑ 6,7-Dimethylribolumazine from *Er. ashbyii*

Fig. 3. X-Ray Diffraction Patterns of 6,7-Dimethylribolumazine from *Er. ashbyii* and Synthesized Product

Comparison of the product with the 6,7-dimethylribolumazine isolated from the mycelium of *Er. ashbyii* showed that they are identical judging not only from their melting point, optical rotation, and Rf values but also from their ultraviolet and infrared spectra, and X-ray diffraction patterns, as shown in Figs. 1, 2, and 3, respectively. Moreover, it was found that the product is able to produce riboflavin by the action of a crude enzyme solution prepared from *Er. ashbyii*, the details of which will be reported elsewhere.

Summary

Attempt was made for the total synthesis of 6,7-dimethylribolumazine which is considered to be an intermediate in the biosynthesis of riboflavin. In the first place, crude 4-ribitylaminouracil was prepared from 4-chlorouracil and D-ribamine. Although this compound could not be produced in a pure enough state, it was isolated as its 5-nitroso derivative as pretty rods, m.p. 105°(decomp.). This nitroso compound was reduced to the corresponding amino compound with sodium hydrosulfite, but as the latter could not be separated pure, it was immediately led to 6,7-dimethylribolumazine by reaction with diacetyl. Physical properties of the product were in complete agreement with those of the 6,7-dimethylribolumazine separated from the mycelium of *Er. ashbyii*, and the structure of the product was thereby positively established.

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