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Stereochemistry of Hydrogenation of (−)-Dehydrogriseofulvin to (+)-Griseofulvin with a Cell-Free System of *Streptomyces cinereocrocatus*

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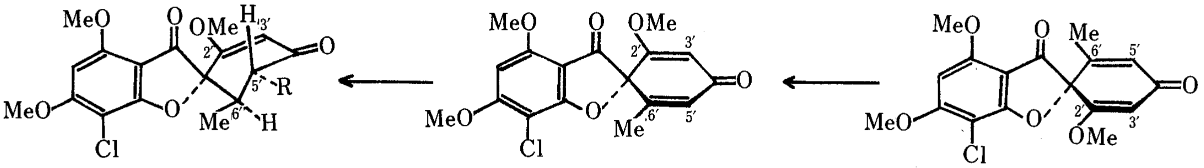
To elucidate the stereochemistry of the hydrogenation of (−)-dehydrogriseofulvin (**1a**) to (+)-griseofulvin (**3a**) by *Streptomyces cinereocrocatus*, we have prepared from the microorganism a cell-free system which can transform **1a** to **3a**. The hydrogenation activity of the cell-free system increased in the presence of added reduced nicotinamide adenine dinucleotide phosphate (NADPH). The stereochemistry of hydrogenation was determined by 400 MHz proton nuclear magnetic resonance (^{1}H-NMR) analysis of the products obtained by the enzymatic conversion of (−)-[5′-^{2}H]-dehydrogriseofulvin (**1b**) and also by the enzymatic conversion of **1a** in medium containing deuterium oxide.

Keywords——cell-free system; *Streptomyces cinereocrocatus*; NADPH; griseofulvin; dehydrogriseofulvin; stereochemistry; hydrogenation; deuterated compound

We have recently shown that (−)- and (+)-dehydrogriseofulvin (**1a** and **2**) are transformed into (+)-griseofulvin (**3a**) by *Streptomyces cinereocrocatus* NRRL 3443.[1] Moreover, deuterium nuclear magnetic resonance (^{2}H-NMR) and mass spectrometric studies clearly demonstrated that the microbial hydrogenation of **1a** proceeds to give (+)-griseofulvin (**3a**), proving that the stereochemistry of the 5′- and 6′-hydrogens of the reduction product is *trans* as regards the two hydrogen atoms introduced.[1a,b] In this continuing series of studies, the stereochemistry of the hydrogenation of (−)-dehydrogriseofulvin (**1a**) in a cell-free system of *S. cinereocrocatus* has been elucidated.



3a: R=H
4: R=OH

1a

2

Chart 1

Results and Discussion

Transformation of (−)-Dehydrogriseofulvin to (+)-Griseofulvin by the Cell-Free System

The harvested mash from *Streptomyces cinereocrocatus* NRRL 3443 was disrupted with an X-press, an instrument which uses the freeze–pressing principle, and centrifuged to yield a supernatant. Its activity was determined by examining the ratio of transformation of (−)-dehydrogriseofulvin (**1a**) to griseofulvin in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) or/and reduced nicotinamide adenine dinucleotide

TABLE I. Effect of Cofactors on the Transformation of (−)-Dehydrogriseofulvin (**1a**) to (+)-Griseofulvin by the Cell-Free System of *Streptomyces cinereocrocatus*

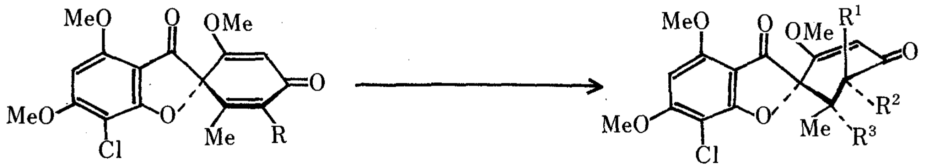
Cofactor	(+)-Griseofulvin formed (%)	(−)-Dehydrogriseofulvin recovered (%)
None	18	80
NADH (2 mg/10 ml)	18	78
NADPH (2 mg/10 ml)	64	36
NADH and NADPH (2 mg each/10 ml)	62	38

(NADH). The results indicated that the cell-free system did hydrogenate **1a** to **3a**, without a requirement for preculture with (−)-dehydrogriseofulvin in order to induce enzymes. Table I indicates the effects of NADPH and/or NADH on the hydrogenation of (−)-dehydrogriseofulvin (**1a**) in the cell-free system. When NADPH[2)] was added to the cell-free system, the transformation activity was increased as compared with that of the control. When NADPH was replaced by NADH, the hydrogenation activity was almost equal to that of the control. However, a cell-free extract heated at 80 °C for 10 min exhibited no activity, even in the presence of added NADPH, indicating that the enzyme extract is heat-labile. The activities of the supernatants of centrifugation at 30000 × *g* and at 100000 × *g* were almost the same. The product obtained from (−)-dehydrogriseofulvin (**1a**) in the presence of NADPH was demonstrated to be (+)-griseofulvin (**3a**) by proton nuclear magnetic resonance (^{1}H-NMR) and circular dichroism (CD) measurements, the results being identical with those for the fermentation product from *S. cinereocrocatus*. The results clearly indicate that NADPH is required for the transformation of (−)-dehydrogriseofulvin (**1a**) to (+)-griseofulvin (**3a**) by the cell-free extract. Further, in order to elucidate whether 5′α-hydroxygriseofulvin (**4**)[1a)] could be formed by the cell-free system, the reaction was attempted essentially under the same conditions as described above except that (+)-griseofulvin was used as a substrate. Analysis of the reaction mixture indicated that no transformation product was formed, showing that the cell-free system contained no enzymatic activity for hydroxylation.

The microbial transformation of (+)-dehydrogriseofulvin (**2**), the enantiomer of (−)-dehydrogriseofulvin, by intact *S. cinereocrocatus* cells has been proved to afford (+)-griseofulvin (**3a**). In this transformation, (+)-dehydrogriseofulvin (**2**) is first converted to its enantiomer, natural (−)-dehydrogriseofulvin (**1a**), followed by enzymatic hydrogenation of the latter.[1b)] In order to elucidate whether **2** could be transformed into **3a** by the above mentioned cell-free system, an examination was performed under the same conditions as described above except that (+)-dehydrogriseofulvin (**2**) was used as a substrate. However, no hydrogenation proceeded, and the starting material was recovered unchanged as demonstrated by ^{1}H-NMR and CD measurements.

Transformation of (−)-[5′-^{2}H]-Dehydrogriseofulvin to (+)-[5′α-^{2}H]-Griseofulvin by a Cell-Free System

Evidence that the hydrogen atom at C-5′ of (−)-dehydrogriseofulvin (**1a**) is retained without being exchanged with hydrogen originating from water in the medium and evidence for the stereochemistry of the hydrogenation of **1a** were provided by analysis of the incubation product of (−)-[5′-^{2}H]-dehydrogriseofulvin (**1b**) with a cell-free system. That is, treatment of (−)-[5′-^{2}H]-dehydrogriseofulvin (**1b**) ($^2H_0$ 74%, $^2H_1$ 26%) in the presence of NADPH afforded a product, whose ^{1}H-NMR spectrum was apparently identical with that of **3a** except for a 24% decrease in the integral of the C-5′α proton signal. This result indicated that the product is (+)-[5′α-^{2}H]-griseofulvin (**3b**), in which the stereochemistry of 5′-^{2}H is identical with that of the fermentation product[1a)] obtained with intact cells.



1a: R=H
1b: R=^{2}H

3b: R^1 and R^3=H, R^2=^{2}H
3c—**e**: R^2=H, R^1 and R^3=^{2}H

1) NADPH, cell-free system
2) NADPH, cell-free system (D_2O)
3) 2)+glucosamine-6-phosphate

Chart 2

Deuterium Incorporation into (+)-Griseofulvin on Incubation with (−)-Dehydrogriseofulvin with a Cell-Free System Containing D_2O

Since the hydrogenation of (−)-dehydrogriseofulvin (**1a**) to (+)-griseofulvin (**3a**) by a cell-free extract of *S. cinereocrocatus* stoichiometrically requires one proton and one hydride, the examination was performed under the same conditions as described above except that 0.03 M phosphate buffer prepared with D_2O was used in order to clarify the origin of two hydrogens on the product. The product was proved to be (+)-[5′β,6′α-^{2}H]-griseofulvin (**3c**) by ^{1}H-NMR analysis (Chart 2). The deuterium incorporations at the 5′β- and 6′α-positions of **3c** were estimated to be 50 and 24%, respectively, on the basis of the integrals of the corresponding proton peaks in the ^{1}H-NMR. This incorporation of deuterium at both the 5′β and 6′α positions suggests that deuteride (D^-), formed during incubation in the cell-free system containing D_2O, participates in the hydrogenation as well as a deuteron (D^+) from D_2O. On the other hand, reducing sugars in the cell-free system were determined to amount to 19 mg per ml of the cell-free extract by the method of Somogyi and Nelson.[3)] Since glucosamine-6-phosphate is known to be an inhibitor of glucose-6-phosphate dehydrogenase,[4)] its sodium salt was added to the cell-free system at concentrations of 5 and 15 mg per ml of the extract in order to eliminate the possibility of the formation of [4-^{2}H]NADPH in the medium containing deuterium oxide, and incubations were performed as described above. The deuterium incorporations into **3d** and **3e** at the 5′β- and 6′α-positions were 51 and 16%, and 50 and 5%, respectively, by ^{1}H-NMR analysis. These results indicated that deuterium incorporation occurred at almost the same rate at 5′β whereas a marked decrease in deuterium incorporation at the 6′ position was observed, suggesting that a deuteron (D^+) is incorporated to give deuterated (+)-griseofulvin with deuterium at the 5′β-position.

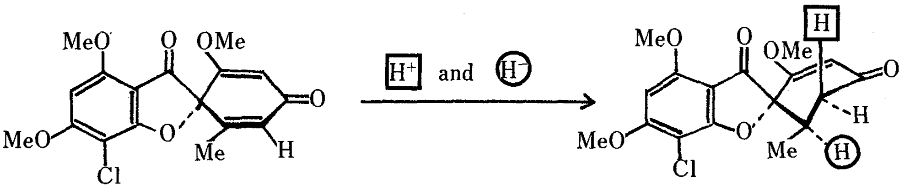


Chart 3

The above results indicate that incubation in the cell-free system of *S. cinereocrocatus* with NADPH added as a cofactor transformed (−)-dehydrogriseofulvin (**1a**) into (+)-griseofulvin (**3a**). However, the same cell-free system lacked the ability to transform (+)-

dehydrogriseofulvin (**2**) into the hydrogenation product, (+)- or (−)-griseofulvin, suggesting that an isomerase, which interconverts (−)- and (+)-dehydrogriseofulvin, is absent in the cell-free system. Moreover, the results indicate that the 5′α- and 5′β-hydrogens of (+)-griseofulvin, the hydrogenation product, originate from 5′-hydrogen of (−)-dehydrogriseofulvin (**1a**) and a proton from the medium, respectively, and the origin of 6′α-hydrogen is a hydride ion donated by the enzyme system(s) (Chart 3).

In summary, NADPH appears to be an effective cofactor in the formation of (+)-griseofulvin from (−)-dehydrogriseofulvin, although its mechanism (whether NADPH participates directly or indirectly) is unclear, and it is suggested that the hydrogen atom incorporated at C-5′ is derived from water in the medium of the cell-free system.

Experimental

Materials——$NADP^+$, NADH, and NADPH were purchased from Boehringer Mannheim GmbH. Glucosamine-6-phosphate sodium salt was purchased from Sigma Chemical Co. Deuterium oxide (purity, 99.8 atom %D) was purchased from Merck Sharp & Dohme Canada Ltd., Montreal, Canada. All other reagents were purchased from commercial sources and were of analytical grade. Synthesis and physical data of (−)- and (+)-dehydrogriseofulvin (**1a** and **2**) and (−)-[5′-^{2}H]-dehydrogriseofulvin (**1b**) ($^2H_0$ 74%, $^2H_1$ 26%) were described in previous papers.[1a,b)]

Apparatus——All melting points were obtained on a micro-melting point apparatus, type MM2 (Shimadzu Seisakusho Ltd.), and are uncorrected. Gas liquid chromatography (GLC) was carried out on a Shimadzu GC-6A gas liquid chromatograph equipped with a flame ionization detector, with nitrogen as the carrier gas. A glass column (2 m × 3 mm i.d.) of 1.5% OV-17 on Chromosorb W was used. ^{1}H-NMR spectra were obtained on a JEOL JNM-GX 400 FT NMR spectrometer. All ^{1}H-NMR spectra were recorded in deuteriochloroform and data are reported as parts per million downfield from Me_4Si ($\delta = 0$). Mass spectra (MS) were recorded on a JEOL D-100 spectrometer at 75 eV ionizing potential and are reported as m/z. Optical rotations were measured on a JASCO DIP-SL automatic polarimeter with a cell of 10-cm light path length, and CD spectra were taken in a 0.5-mm cell at room temperature (24—25 °C) in chloroform on a JASCO J-20 recording spectropolarimeter. Column chromatography was performed with Kanto Kagaku silica gel (100 mesh). pH values were recorded on a LAB-O-MATE (Beckman-Toshiba, Ltd.).

Preparation of the Cell-Free System, Typical Incubation Conditions (TIC) and Separation of Product——The microorganism was grown on the medium described previously.[1b)] *Streptomyces cinereocrocatus* NRRL 3443 was maintained on Glucose-Czapeck-agar slants. A nutrient solution consisting of 30 g of cornstarch, 20 g of molasses, 40 g of cornsteep liquor, 10 g of calcium carbonate and 2.5 g of yeast extract per liter of distilled water was distributed in 12 flasks (20 ml/flask). The contents were autoclaved at 12 °C for 20 min. Each flask was inoculated with a 5-mm plug of mycelium of *S. cinereocrocatus* from the slants. The flasks were then incubated for 48 h at 28 °C and 100 rpm in an incubator shaker. Sakaguchi flasks (500 ml) containing 100 ml of sterile medium were inoculated with 8 ml of 48 h-old *S. cinereocrocatus* culture and incubated for an additional 60 h. The mycelium was harvested by centrifugation at 3000 rpm for 10 min and washed with cold 0.03 M phosphate buffer (pH 7.0). The cell-free system was obtained by disruption of the wet mycelium (*ca.* 25 g) of *S. cinereocrocatus* by three passages through the X-press (Biotec Inc., U.S.A.), and by addition of two volumes of 0.03 M phosphate buffer. The crushed cell mass was centrifuged immediately at 30000 × *g* for 20 min. Centrifugations were carried out at 4 °C. The supernatant (*ca.* 33 ml) was obtained by decantation and used directly as the crude enzyme source. A solution of a substrate (1 mg) in acetone (150 μl) was added to a mixture of coenzyme (2 mg) and cell-free extract (10 ml). The whole was incubated at 28 °C with continuous shaking for 5 h, then extracted with chloroform. The extract was washed with 5% Na_2CO_3 and concentrated under reduced pressure to give a residue. The transformation product (griseofulvin) and recovered substrate (dehydrogriseofulvin) were quantitated by GLC of the residue.

Determination of Protein——Protein concentration of the cell-free extract was determined by the method of Lowry *et al.*[5)] with bovine serum albumin as a standard. All of the cell-free extracts contained 15—18 mg protein/ml.

Determination of Reducing Sugars——Concentration of reducing sugars in the cell-free extract was measured by the method of Somogyi and Nelson.[3)]

Transformation of (−)-Dehydrogriseofulvin (1a) to (+)-Griseofulvin (3a) by a Cell-Free System——(−)-Dehydrogriseofulvin (**1a**) (30 mg) and NADPH (60 mg) were added to 200 ml of the cell-free system, and the mixture was incubated under typical incubation conditions (TIC; described above). The yields of griseofulvin and recovered dehydrogriseofulvin are shown in Table I. The residue from a cell-free experiment in benzene was subjected to column chromatography on silica gel (20 g). 1) Elution with benzene–methylene chloride (60 : 40) and recrystallization of the product from benzene gave (+)-griseofulvin (**3a**), mp 219—220 °C, $[\alpha]_D^{24}$ +336 ° ($c = 0.10$, $CHCl_3$). The ^{1}H-NMR and MS were identical with those of a standard sample of (+)-griseofulvin. Molecular ellipticity $[\theta]$ ($c = 1.0$ mg/ml): $[\theta]_{352}$

0, $[\theta]_{323}$ +20250, $[\theta]_{292}$ +32990, $[\theta]_{268}$ +4450, $[\theta]_{237}$ +116460. 2) The fraction obtained by column chromatography corresponding to recovered substrate was recrystallized from benzene to give (−)-dehydrogriseofulvin (**1a**), mp 273—275 °C, $[\alpha]_D^{24}$ −35.2° (c=0.24, $CHCl_3$). The ^{1}H-NMR and MS were identical with those of (−)-dehydrogriseofulvin (**1a**). Molecular ellipticity $[\theta]$ (c=1.0 mg/ml): $[\theta]_{302}$ −21290, $[\theta]_{290}$ 0, $[\theta]_{282}$ +19270, $[\theta]_{263}$ 0, $[\theta]_{258}$ −2820, $[\theta]_{255}$ 0, $[\theta]_{246}$ +13660, $[\theta]_{242}$ 0, $[\theta]_{235}$ −25560, $[\theta]_{228}$ 0.

Incubation of (+)-Dehydrogriseofulvin (2) by a Cell-Free System——The incubation and separation were carried out essentially as described above (TIC and separation of product) except that 10 mg of (+)-dehydrogriseofulvin (**2**) was used as a substrate and 20 mg of NADPH was added to the cell-free system (100 ml). The residue from the incubation was subjected to column chromatography on silica gel (10 g). Elution with benzene–methylene chloride (60 : 40) and recrystallization from benzene gave (+)-dehydrogriseofulvin (**2**), mp 274—275 °C, $[\alpha]_D^{24}$ +36.8 °C (c= 0.27, $CHCl_3$). The ^{1}H-NMR and MS were identical with those of a standard sample of (+)-dehydrogriseofulvin. Molecular ellipticity $[\theta]$ (c=1.0 mg/ml): $[\theta]_{302}$ +21300, $[\theta]_{290}$ 0, $[\theta]_{282}$ −19290, $[\theta]_{263}$ 0, $[\theta]_{258}$ +2890, $[\theta]_{255}$ 0, $[\theta]_{246}$ +13590, $[\theta]_{242}$ 0, $[\theta]_{235}$ −25520, $[\theta]_{228}$ 0.

Incubation of (+)-Griseofulvin (3a) with the Cell-Free System——The incubation and separation were carried out essentially as described above (TIC and separation of product) except that 10 mg of (+)-griseofulvin (**3a**) was used as a substrate and 20 mg of NADPH was added to the cell-free system (100 ml). The residue from the incubation was found to be the starting material (**3a**, 100% recovery) by GLC and ^{1}H-NMR analyses.

Transformation of (−)-[5′-^{2}H]-Dehydrogriseofulvin (1b) to (+)-[5′α-^{2}H]-Griseofulvin (3b) by a Cell-Free System——The incubation and separation were carried out essentially as described above (TIC and separation product) except that 15 mg of (−)-[5′-^{2}H]-dehydrogriseofulvin (**1b**) was used as a substrate and 30 mg of NADPH was added to the cell-free system (150 ml). The residue from the incubation was found to consist of griseofulvin (62% yield) and dehydrogriseofulvin (38% recovery) by GLC analysis. Column chromatography of the residue on silica gel (20 g) and recrystallization from benzene gave a mixture (48 : 52) of (+)-[5′α-^{2}H]-griseofulvin (**3b**) and (−)-[5′-^{2}H]-dehydrogriseofulvin (**1b**). The ^{1}H-NMR spectrum of **3b** was almost the same as that of **3a** except for a decrease to 0.76 H in the integral of the 5′α proton signal (2.40—2.46 ppm).

Transformation of (−)-Dehydrogriseofulvin (1a) to (+)-[5′β,6′α-^{2}H]-Griseofulvin (3c) by a Cell-Free System Containing D_2O——All of the experiments were carried out essentially as described above (TIC and separation of product) except that 0.03 M phosphate buffer prepared with D_2O was used as a buffer, and 26 mg of NADPH and 13 mg of **1a** were added to the cell-free system (130 ml). The residue from the incubation was found to consist of griseofulvin (49% yield) and dehydrogriseofulvin (51% recovery) by GLC analysis. Column chromatography of the residue on silica gel (10 g) and recrystallization from benzene gave a mixture (45 : 55) of (+)-[5′β,6′α-^{2}H]-griseofulvin (**3c**) and **1a**. The ^{1}H-NMR spectrum of **3c** was almost the same as that of **3a** except for decreases to 0.50 H and 0.76 H in the integrals of the 5′β and 6′α proton signals (3.03—3.07 and 2.83—3.00 ppm), respectively.

Transformation of (−)-Dehydrogriseofulvin (1a) into 5′,6′-Deuterated (+)-Griseofulvin (3d and 3e) by a Cell-Free System Containing D_2O in the Presence of Glucosamine-6-phosphate——1) All of the experiments were carried out essentially as described above (TIC and separation of product) except that 500 mg of glucosamine-6-phosphate sodium salt and 40 mg of NADPH were added to the cell-free system (100 ml). The residue was found to consist of griseofulvin (49% yield) and dehydrogriseofulvin (51% recovery) by GLC analysis. Column chromatography of the residue from the incubation on silica gel (10 g) and recrystallization of the product from benzene gave a mixture (42 : 58) of **3d** and **1a**. The ^{1}H-NMR spectrum of **3d** was almost the same as that of **3a** except for decreases to 0.49H and 0.84H in the integrals of the 5′β and 6′α proton signals (3.03—3.07 and 2.83—3.00 ppm), respectively. 2) All of the experiments were carried out essentially as described above except that 1.1 g of glucosamine-6-phosphate sodium salt was added to the cell-free system (70 ml). The residue was found to consist of griseofulvin (40% yield) and dehydrogriseofulvin (51% recovery) by GLC analysis. Column chromatography of the residue from the incubation on silica gel (10 g) and recrystallization of the product from benzene gave a mixture (52 : 48) of **3e** and **1a**. The ^{1}H-NMR spectrum of **3e** was almost the same as that of **3a** except for decrease to 0.50 H and 0.95 H in the integrals of the 5′β and 6′α proton signals, respectively.

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