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Stereospecific Incorporation of Hydrogen from Reduced Nicotinamide Adenine Dinucleotide Phosphate in Hydrogenation of (−)-Dehydrogriseofulvin to (+)-Griseofulvin by a Partially Purified Enzyme System of *Streptomyces cinereocrocatus*

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The stereochemistry of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent hydrogenation was investigated by the conversion of (−)-dehydrogriseofulvin (**1**) to (+)-griseofulvin (**2a**) by the use of a partially purified enzyme system of *Streptomyces cinereocrocatus* in the presence of (4*R*)- or (4*S*)-[4-$^{2}H_{1}$]NADPH. The results of 270 MHz proton nuclear magnetic resonance spectroscopy indicated that the origin of the 6′α-hydrogen of **2a** is a hydride ion donated by pro-4*R*-hydrogen of NADPH.

Keywords——*Streptomyces cinereocrocatus*; NADPH; (4*R*)-[4-$^{2}H_{1}$]NADPH; (4*S*)-[4-$^{2}H_{1}$]-NADPH; griseofulvin; dehydrogriseofulvin; hydrogenation; enzyme system

The importance of microbial transformation of natural products has been emphasized in many excellent reviews,[1] and the microbial transformation of (−)-dehydrogriseofulvin (**1**) to (+)-griseofulvin (**2a**) was initially investigated by Andres and his co-workers[2] using *Streptomyces cinereocrocatus* NRRL 3443 (Chart 1). Since then, we have successfully elucidated the stereochemistry of the microbial reduction by ^{2}H nuclear magnetic resonance spectroscopy.[3] In the preceding paper of this series,[4] we prepared from the microorganism, *Streptomyces cinereocrocatus*, a cell-free system which can transform (−)-dehydrogriseofulvin (**1**) to (+)-griseofulvin (**2a**), in order to elucidate the mechanism of the hydrogenation. The assumption that reduced nicotinamide adenine dinucleotide phosphate (NADPH) might be a cofactor in the hydrogenation was confirmed by the experiments, which showed an increment of the transformation ratio by NADPH added to an incubation mixture containing the cell-free system. The stereochemistry of hydrogenation was demonstrated by proton nuclear magnetic resonance (^{1}H-NMR) analysis of the products which were obtained by the conversion of (−)-[5′-^{2}H]dehydrogriseofulvin and also of **1** in a medium containing deuterium oxide. The results indicated that the 5′α- and 5′β-hydrogens of (+)-griseofulvin originate from 5′-hydrogen of (−)-dehydrogriseofulvin and a proton from the medium, respectively, and the origin of 6′α-hydrogen is a hydride ion donated by the enzyme system(s). Therefore, NADPH appeared to be an effective cofactor in the formation of (+)-griseofulvin

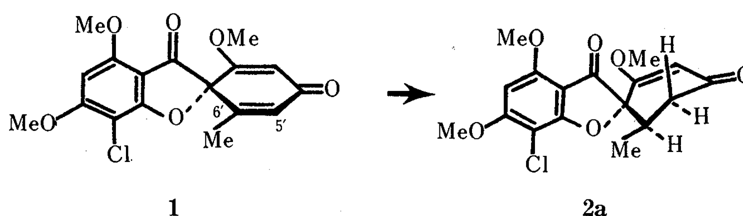

Chart 1

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(**2a**) from **1**, although the mechanism (whether NADPH participates directly or indirectly) was unclear.

In this paper, we show that the origin of 6′α-hydrogen of **2a** is a hydride ion donated by pro-4*R*-hydrogen of NADPH.

Results and Discussion

Syntheses of (4*R*)- and (4*S*)-[4-$^{2}H_{1}$]NADPH and [6′α-$^{2}H_{1}$]Griseofulvin as the Standard Sample

In the preceding paper,[4] we proved that NADPH is an effective cofactor in the formation of (+)-griseofulvin (**2a**) from **1** in a cell-free system of *Streptomyces cinereocrocatus*. In order to understand the reaction mechanism more fully, two forms of NADPH labelled stereospecifically with deuterium were indispensable. Consequently, (4*R*)- and (4*S*)-[4-$^{2}H_{1}$]NADPH were synthesized from $NADP^{+}$ according to the method of Cornforth *et al.*[5] The isotopic and stereochemical purities of the selectively labelled compounds were investigated by 270 MHz ^{1}H-NMR spectroscopy.[6] ^{1}H-NMR analyses of the (4*R*)- and (4*S*)-[4-$^{2}H_{1}$]NADPH confirmed that the extent of deuteration was greater than 98% (Fig. 1).

On the other hand, [6′α-^{2}H]griseofulvin (**2b**, $^{2}H_{0}$ 13%, $^{2}H_{1}$ 85%, $^{2}H_{2}$ 2%) as a standard sample was prepared by treatment with neutral alumina of [5′α,6′α-^{2}H]griseofulvin (**2c**) ($^{2}H_{0}$ 5.5%, $^{2}H_{1}$ 24.0%, $^{2}H_{2}$ 70.5%), which was obtained by catalytic deuteration of (−)-dehydrogriseofulvin (**1**) (Chart 2). Deuterium isotopic shifts were observed in the ^{1}H-NMR spectrum (refer to the experimental section for details). Parts of the ^{1}H-NMR spectrum of **2b** are shown in Fig. 2b; the intensity of the 6′α-H signal decreased to 0.13H and the 5′β- and 5′α-H signals appeared almost as doublets and the 6′-methyl signal almost as a singlet.

Incubation of (−)-Dehydrogriseofulvin in the Presence of (4*R*)- and (4*S*)-[4-$^{2}H_{1}$]NADPH as a Cofactor with a Cell-Free System

To elucidate the stereochemistry of NADPH-dependent hydrogenation, the reaction was performed in the presence of (4*R*)- or (4*S*)-[4-$^{2}H_{1}$]NADPH instead of undeuterated NADPH

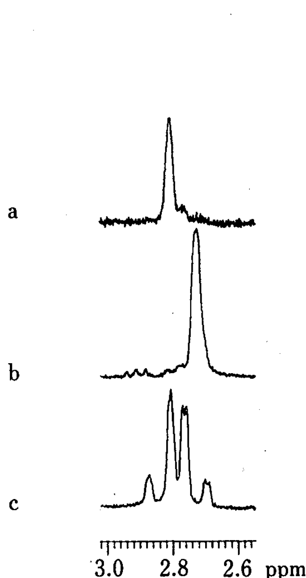

Fig. 1. The 270 MHz ^{1}H-NMR Signals from Position 4 of the Nicotinamide Moiety of NADPH and Deuterated NADPH

a, (4*S*)-[4-$^{2}H_{1}$]NADPH; b, (4*R*)-[4-$^{2}H_{1}$]NADPH; c, undeuterated NADPH.

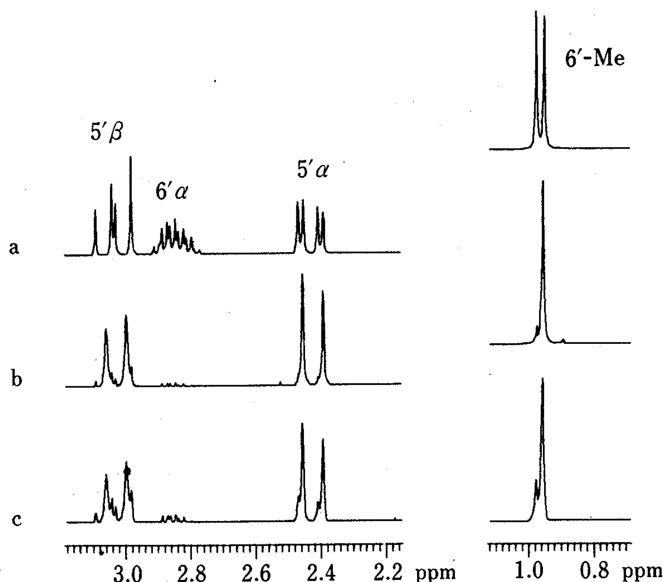

Fig. 2. Partial 270 MHz ^{1}H-NMR Spectra of **2a**, **2b**, and the Transformation Product (**2d**)

a, **2a**; b, **2b**; c, **2d**.

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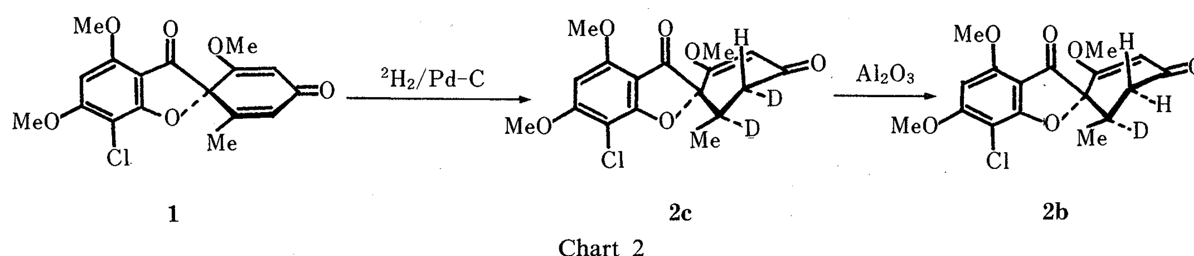

Chart 2

Table I. Partially Purified Enzyme System

Enzyme fraction	Protein (mg/ml)	Activity (units)	Specific activity (units/mg protein)	Purification (fold)
Cell-free extract	23.5	4760	1.45	1
DEAE-Sephacel	1.0	513	20.5	14.7

in the standard cell-free system[4] of *S. cinereocrocatus*. In each case, the transformation product was proved to be undeuterated (+)-griseofulvin, as determined by ^{1}H-NMR and mass spectrometric analyses. The results indicate that the cell-free system contains endogenous NADPH and moreover, hydrogen exchange between deuterated NADPH and the system takes place under the reaction conditions. Therefore, we attempted to prepare a partially purified enzyme system from the standard cell-free system.

Preparation of a Partially Purified Enzyme System

The results of purification of the standard cell-free system are summarized in Table I. Exclusion of endogenous NADPH was performed at the step of diethylaminoethyl (DEAE)-Sephacel chromatography, which afforded an enzyme system with a specific activity of 20.5 U/mg protein (refer to the experimental section for enzymatic activity determination).

Incubation of (−)-Dehydrogriseofulvin in the Presence of (4*R*)- or (4*S*)-[4-^{2}H]NADPH as a Cofactor with the Partially Purified Enzyme System

Deuterium-labelled NADPH has been used in the elucidation of the stereochemical course of enoyl reduction catalyzed by fatty acid synthetase[7] and also of folic acid by dihydrofolate reductase.[8] Many attempts have been made to provide a rational explanation for the stereochemical preferences of nicotinamide adenine dinucleotide- (NAD-) or NADP-requiring dehydrogenases, but they remain "as intractable as ever and have yet to yield the secrets of their underlying principles."[9]

To elucidate the stereoselectivity of NADPH-dependent hydrogenation, the partially purified enzyme system was incubated with (−)-dehydrogriseofulvin in the presence of added undeuterated NADPH or (4*R*)- or (4*S*)-[4-$^2H_1$]NADPH. The results are summarized in Table II. Although the yield (30%) in the presence of undeuterated NADPH was almost the same as that (26%) in the presence of (4*S*)-[4-$^2H_1$] NADPH, the yield in the presence of (4*R*)-[4-$^2H_1$]NADPH was 7%, which suggested deuterium incorporation into the product, since direct deuteride transfer is subject to the isotopic effect. Thus, the product and the recovered (−)-dehydrogriseofulvin were separated by high-performance liquid chromatography (HPLC). ^{1}H-NMR analysis showed that when undeuterated or (4*S*)-[4-$^2H_1$]NADPH was added, the product was undeuterated (+)-griseofulvin (**2a**). However, the product obtained with (4*R*)-[4-$^2H_1$]NADPH was deuterated ($^2H_0$ 28%, $^2H_1$ 72%) and was proved to be [6′α-$^2H_1$]griseofulvin (**2d**) by ^{1}H-NMR comparison with a standard sample (**2b**) (Fig. 2), demonstrating that the origin of 6′α-hydrogen is a hydride ion donated by pro-4*R*-hydrogen of NADPH, in which the configuration of pro-4*R*-hydrogen would be axial.[10]

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Table II. Effects of NADPH and (4*R*)-[4-$^2H_1$]- and (4*S*)-[4-$^2H_1$]NADPH on the Transformation of (−)-Dehydrogriseofulvin (DGF) to (+)-Griseofulvin (GF) by a Partially Purified Enzyme System of *S. cinereocrocatus*

NADPH added	(+)-GF formed (%)	(−)-DGF recovered (%)
None	—	100
NADPH	30	70
(4*R*)-[4-$^2H_1$]NADPH	7	93
(4*S*)-[4-$^2H_1$]NADPH	26	74

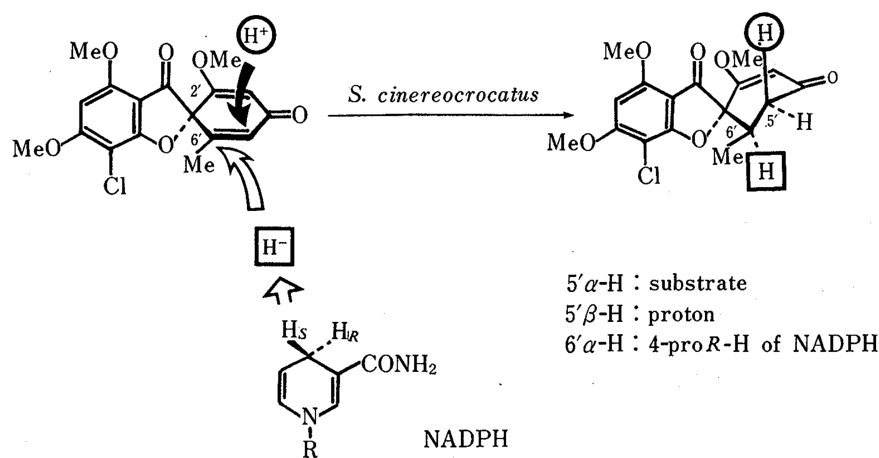

Chart 3

Thus, it has been unequivocally demonstrated that in the microbial reduction of (−)-dehydrogriseofulvin (**1**) to give (+)-griseofulvin (**2a**), the 5′α- and 5′β-hydrogens of **2a** originate from 5′-hydrogen of **1** and a proton from the incubation medium, respectively, and also that the origin of the 6′α-hydrogen of **2a** is a hydride ion donated by pro-4*R*-hydrogen of NADPH (Chart 3).

Experimental

Apparatus——All melting points were obtained on a Shimadzu MM2 micro-melting point apparatus, and are uncorrected. ^{1}H-NMR spectra were obtained at 270 MHz on a JEOL JNM-GX 270 FT NMR spectrometer. All ^{1}H-NMR data were recorded in deuteriochloroform or D_2O and are reported as parts per million downfield from Me_4Si ($\delta = 0$) or sodium trimethylsilylpropionate-2,2,3,3-d_4 (TSP), respectively. Abbreviations used: s = singlet, d = doublet, br = broad, m = multiplet, dd = doublet of doublets, q = quartet. Mass spectra (MS) were recorded on a JEOL D-100 spectrometer at 75 eV ionizing potential. Column chromatography was performed with Kanto Kagaku silica gel (100 mesh). HPLC was performed on a Bondapak NH2 column (3.9 mm × 30 cm), using a Waters pump (model 510) and a Waters detector (model 480 spectrophotometer, set at 254 nm). *n*-Hexane–isopropyl alcohol (90 : 10, v/v) was used as an eluent. Gas liquid chromatography (GLC) was carried out on a Shimadzu GC-6a gas liquid chromatograph by using 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. pH values were recorded on a LAB-O-MATE (Beckman-Toshiba, Ltd.).

Materials——$NADP^+$, NADPH and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim. Isocitrate dehydrogenase was purchased from Sigma Chemical Co. All other reagents were purchased from commercial sources and were of analytical grade.

(−)-Dehydrogriseofulvin (1)——Synthesis of (−)-dehydrogriseofulvin (**1**) was performed as described in a previous paper.[2b)]

[6′α-^{2}H]Griseofulvin (2b)——A suspension of 5% palladium–charcoal catalyst (135 mg) in an ethyl acetate solution (200 ml) of (−)-dehydrogriseofulvin (**1**) (900 mg) was shaken under a stream of deuterium at atmospheric pressure and at room temperature. The hydrogenation was stopped after 90 min. The catalyst was removed by

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filtration and the filtrate was concentrated *in vacuo*. The residue was dissolved in chloroform and washed with 2 N aqueous sodium hydroxide. The neutral material was washed with water, dried (Na_2SO_4) and concentrated *in vacuo* (yield, 627 mg). The neutral crude product in benzene was chromatographed on silica gel and eluted with two solvent mixtures. Elution with benzene–methylene chloride (20:80) gave a 1:1 mixture (480 mg) of the product and the starting material (GLC analysis). The mixture (480 mg) in acetic acid (40 ml) was stirred with 2.5 g of zinc dust for 30 min at room temperature, then the zinc was filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in chloroform and the solution was washed with 1 N sodium hydroxide and water, dried ($MgSO_4$) and concentrated *in vacuo* (yield, 188 mg). The neutral product in benzene was chromatographed on silica gel (20 g) and eluted with two solvent mixtures. Elution with benzene–methylene chloride (20:80) and recrystallization of the product from benzene gave (+)-[5′α,6′α-^{2}H]griseofulvin (140 mg) (**2c**) as colorless needles, mp 220 °C. MS: $^2H_0$ 5.5%, $^2H_1$ 24.0%, $^2H_2$ 70.5%. ^{1}H-NMR δ (ppm): 0.955 (2.7H, s, 6′-CH_3, 6′α,5′α-^{2}H), 0.965 (0.3H, d, J=6.60 Hz, 6′-CH_3), 2.423 (0.1H, br d, J=16.83 Hz, 5′α-H, 6′α,5′α-^{2}H), 2.430 (0.03H, dd, J=16.42, 3.96 Hz, 5′α-H), 2.852 (0.1H, m, 6′α-H), 3.007 (0.9H, s, 5′β-H, 6′α,5′α-^{2}H), 3.025 (0.1H, br d, J=16.83 Hz, 5′β-H, 6′α,5′α-^{2}H), 3.033 (0.03H, dd, J=16.42, 13.37 Hz, 5′β-H), 3.61 (3H, s, 2′-OCH_3), 3.97 (3H, s, 4-OCH_3), 4.02 (3H, s, 6-OCH_3), 5.53 (1H, br s, 3′-H), 6.12 (1H, s, 5-H).

A solution of **2d** (130 mg) in chloroform (10 ml) was mixed with neutral alumina (activity II). The mixture was shaken at room temperature for 48 h, then the alumina was filtered off and washed with chloroform. Removal of the combined solvent *in vacuo* gave a white powder (100 mg). MS: $^2H_0$ 10%, $^2H_1$ 63%, $^2H_2$ 27%. The powder was reacted under the above conditions four times, and the resulting white powder (54 mg) in benzene was chromatographed on silica gel and eluted with two solvent mixtures. Elution with benzene–methylene chloride (20:80) and recrystallization from benzene gave colorless plates of [6′α-^{2}H]griseofulvin (30 mg) (**2b**), mp 220 °C. MS: $^2H_0$ 13%, $^2H_1$ 85%, $^2H_2$ 2%. ^{1}H-NMR δ (ppm): 0.955 (2.6H, s, 6′-CH_3, 6′α-^{2}H), 0.965 (0.4H, d, J=6.60 Hz, 6′-CH_3), 2.423 (0.9H, br d, J=16.83 Hz, 5′α-H, 6′α-^{2}H), 2.430 (0.1H, dd, J=16.42, 3.96 Hz, 5′α-H), 2.852 (0.1H, m, 6′α-H), 3.025 (0.9H, br d, J=16.83 Hz, 5′β-H), 3.033 (0.1H, dd, J=16.42, 13.37 Hz, 5′β-H), 3.61 (3H, s, 2′-OCH_3), 3.97 (3H, s, 4-OCH_3), 4.02 (3H, s, 6-OCH_3), 5.53 (1H, br s, 3′-H), 6.12 (1H, s, 5-H).

(4*R*)- and (4*S*)-[4-$^2H_1$]NADPH——Two specimens of specifically deuterium-labelled NADPH were prepared by reduction of 4-deuterio $NADP^+$ with glucose 6-phosphate dehydrogenase (deuterium is located at the 4*R*-position of NADPH) and isocitrate dehydrogenase (deuterium is located at the 4*S*-position of NADPH).[4,5] Each deuterium-labelled NADPH was purified by DEAE-Sephacel column chromatography. Partial ^{1}H-NMR spectra of undeuterated NADPH and (4*R*)- and (4*S*)-[4-$^2H_1$]NADPH are shown in Fig. 1.[10]

Preparation of a Partially Purified Enzyme System——Fermentation and preparation of a cell-free system of *S. cinereocrocatus* were essentially the same as described in previous papers[3] except that 0.03 M phosphate buffer (pH 7.0) contained 0.4 mM dithiothreitol. A solution of 100 mg of protamine sulfate in 2 ml of water was added to a cell-free system (100 ml), and the precipitate formed was removed by centrifugation at 23000 × ***g*** for 30 min. Solid $(NH_4)_2SO_4$ was added to the supernatant and the protein precipitate obtained at 70% saturation was recovered by centrifugation at 23000 × ***g*** for 30 min and dissolved in two volumes of 0.03 M phosphate buffer (pH 7.0). The solution was passed through a Sephadex G-25 column which had been equilibrated with the same 0.03 M phosphate buffer. The Sephadex G-25 treated solution was applied to a DEAE-Sephacel column (3.5 cm × 20 cm) equilibrated with 0.03 M phosphate buffer. The enzyme was eluted with 800 ml of a linear gradient of 0.05—0.50 M KCl in 0.03 M phosphate buffer.

Enzyme Activity——A solution of (−)-dehydrogriseofulvin (**1**) (0.1 mg) in acetone (15 μl) was added to a mixture of NADPH (0.2 mg) and the enzyme solution (1 ml). The whole was incubated at 28 °C with continuous shaking for 2 h, and extracted with chloroform. The extract was washed with 5% Na_2CO_3 and concentrated under reduced pressure to give a residue. One unit (U) of enzymatic activity was defined as the amount of enzyme producing 1 μg of griseofulvin under the above incubation conditions, as analyzed by GLC. Specific activity was expressed as U/mg protein.

Determination of Protein——The protein concentration of each enzyme system was determined by the method of Lowry *et al.*[11] with bovine serum albumin as a standard.

Incubation of (−)-Dehydrogriseofulvin in the Presence of Undeuterated NADPH or (4*S*)-[4-$^2H_1$]NADPH as a Cofactor with the Partially Purified Enzyme System——1) (−)-Dehydrogriseofulvin (**1**) (15 mg) and NADPH (30 mg) were added to 100 ml of the partially purified enzyme system, and the mixture was incubated under the same conditions as used for the enzyme activity test described above. The yields of griseofulvin and recovered dehydrogriseofulvin are shown in Table II. The residue from the partially purified enzyme system in benzene was subjected to column chromatography on silica gel (15 g). Elution with benzene–methylene chloride (60:40) and recrystallization of the product from benzene gave (+)-griseofulvin (**2a**). The ^{1}H-NMR and MS were identical with those of a standard sample of (+)-griseofulvin. 2) All of the experiments were carried out in essentially the same way as described above except that (4*S*)-[4-$^2H_1$]NADPH was used as a cofactor. The yields of griseofulvin and recovered dehydrogriseofulvin are shown in Table II. The residue from the partially purified enzyme system in benzene was subjected to column chromatography on silica gel (15 g). Elution with benzene–methylene chloride (60:40) and recrystallization of the product from benzene gave (+)-griseofulvin (**2a**). The ^{1}H-NMR and MS were identical with

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those of a standard sample of (+)-griseofulvin.

Incubation of (−)-Dehydrogriseofulvin in the Presence of (4*R*)-[4-$^2H_1$]NADPH as a Cofactor with the Partially Purified Enzyme System——All of the experiments were carried out in essentially the same way as described above except that (4*R*)-[4-$^2H_1$]NADPH was used as a cofactor. The yields of griseofulvin and recovered dehydrogriseofulvin are shown Table II. The residue from the partially purified enzyme system in benzene was subjected to column chromatography on silica gel (15 g). Elution with benzene–methylene chloride (60:40) gave a mixture (1:1) consisting of griseofulvin and dehydrogriseofulvin. Griseofulvin was isolated by HPLC with *n*-hexane–isopropyl alcohol (90:10) as an eluent. Recrystallization from benzene gave colorless plates of [6′α-^{2}H]griseofulvin (**2d**). MS: $^2H_0$ 28%, $^2H_1$ 72%. Parts of the ^{1}H-NMR spectrum of **2d** are shown in Fig. 2c and were almost the same as those of **2b**.

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