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Stereochemistry of Transformation of 5′-Formylgriseofulvin to 5′α-Hydroxymethylgriseofulvin with a Cell-Free System of *Streptomyces cinereocrocatus*

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The enzymatic hydrogenation of 5′-formylgriseofulvin (**3a**) with a cell-free system of *Streptomyces cinereocrocatus* afforded 5′α-hydroxymethylgriseofulvin (**4a**). The structure of **4a** was determined by comparison of the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, mass spectrum (MS), circular dichroism (CD) and ultraviolet (UV) spectrum with those of 5′α- and 5′β-hydroxymethylgriseofulvin (**4a** and **5a**) which were synthesized chemically. The stereochemistry of hydrogenation was unequivocally determined by 270 MHz ^{1}H-NMR analysis of the acetonide (**10b**) which was derived from the product obtained by the enzymatic conversion of [7′-^{2}H]5′-formylgriseofulvin (**3b**) (Chart 3). The results clearly indicated that the 7′-*pro-S*-hydrogen of **4a** originates from the 7′-hydrogen atom of the substrate (**3a**), and it was further proved that the 5′β-hydrogen of **4a** originates from the medium by the use of deuterium oxide.

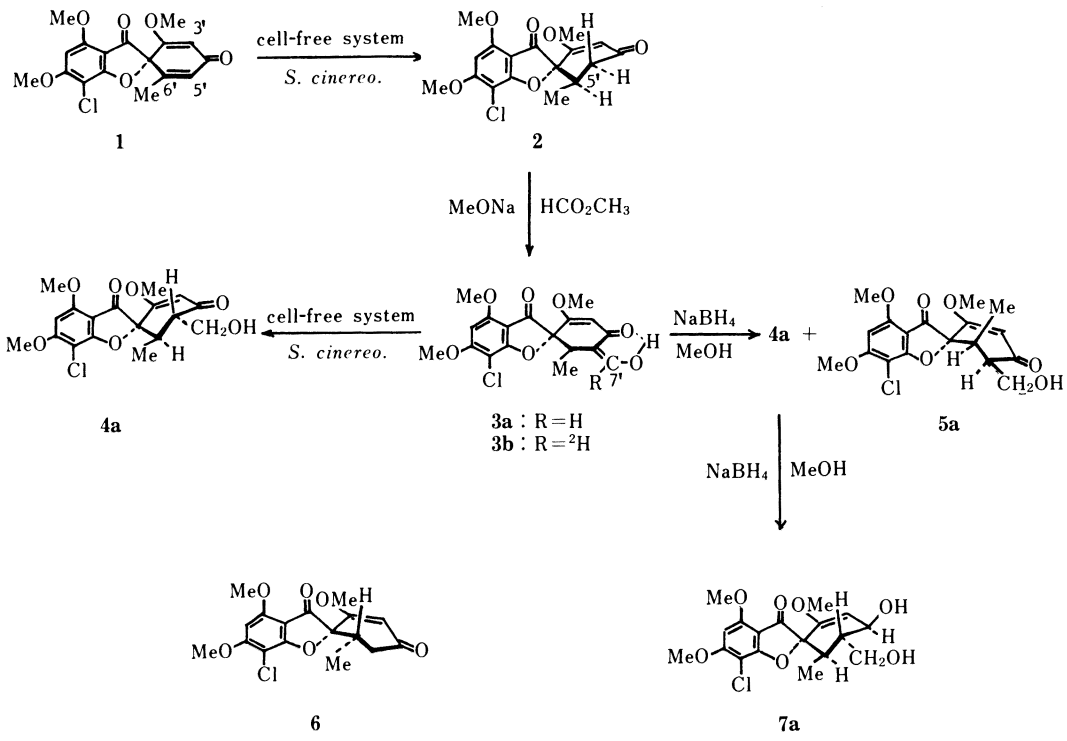
Keywords——cell-free system; *Streptomyces cinereocrocatus* NRRL 3443; 5′-formylgriseofulvin; 5′α-hydroxymethylgriseofulvin; stereochemistry

The microbial transformation[1] of (−)-dehydrogriseofulvin and its derivatives by *Streptomyces cinereocrocatus* NRRL 3443[2] has been investigated in our laboratory.[3] In this work, in order to survey the enzymatic capacity of *S. cinereocrocatus* and to apply the activity synthetically, we examined the reductive transformation of 5′-formylgriseofulvin to 5′α-hydroxymethylgriseofulvin by the cell-free system of the above organism, demonstrating that the enzymatic reduction proceeded stereospecifically.

Results and Discussion

Enzymatic Transformation of 5′-Formylgriseofulvin by a Cell-Free System of *Streptomyces cinereocrocatus* (Chart 1)

Since the cell-free system from *S. cinereocrocatus* can transform (−)-dehydrogriseofulvin (**1**) to (+)-griseofulvin (**2**), we attempted to apply the enzymatic transformation to 5′-formylgriseofulvin (**3a**), which is different from the dienone-type derivative (**1**) in structure. 5′-Formylgriseofulvin (**3a**) was prepared by the method of Newman and Fields.[4] The incubation conditions for the transformation experiment were the same as described in the previous paper,[3d] and the transformation product (**4a**) (mp 211—212 °C) was separated by column chromatography on silica gel. The structure of the product was determined to be 5′α-hydroxymethylgriseofulvin (**4a**) by comparison of the mass spectrum (MS), proton nuclear magnetic resonance (^{1}H-NMR), ultraviolet (UV), and circular dichroism (CD) data with those of the standard compounds (**4a** and **5a**) which are described in the next section. In the enzymatic transformation of 5′-formylgriseofulvin (**3a**), only **4a** was formed stereospecifically, with no **5a**. When **1** was used as the substrate, the enzymatic activity was enhanced by the



1 2 4a 5a 6 7a

Chart 1

addition of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the cell-free system, but no appreciable increase in the reductive activity toward **3a** was observed on the addition of NADPH and/or reduced nicotinamide adenine dinucleotide (NADH).

In order to compare the enzymes which transform 5′-formylgriseofulvin (**3a**) and (−)-dehydrogriseofulvin (**1**), **3a** was incubated with the same partially purified enzyme as described in the previous report.[3e] No transformation was observed, indicating that the enzyme which transforms **3a** differs from that for **1**.

Syntheses of Standard Compounds for Structural Elucidation of the Enzymatic Transformation Product

In order to determine the structure of the enzymatic transformation product, the reduction of 5′-formylgriseofulvin with sodium borohydride in methyl alcohol was undertaken, giving **4a**, **5a**, and **7a**. The molecular weights of two products (**4a** and **5a**) were consistent with dihydrogenation of the starting material (**3a**) by MS analysis. In the ^{1}H-NMR spectra of **4a** and **5a**, the signals at 13.95 and 7.10 ppm due to 7′-OH and 7′-H of **3a** disappeared and new proton signals were observed in the 2.5—4.3 ppm region, suggesting that **4a** and **5a** might be stereoisomers of 5′-hydroxymethylgriseofulvin. When the signal pattern of the 6′α-proton region of **4a** was compared with that of **5a**, the coupling constants (12.6 and 6.6 Hz) in the case of **4a** were larger than those (7.2 and 4.6 Hz) of **5a**. Moreover, the CD spectra (Fig. 1) of **4a** and **5a** were almost the same as those of (+)-griseofulvin (**2**) and (−)-epigriseofulvin (**6**), respectively. Thus, the structures of **4a** and **5a** were determined as 5′α- and 5′β-hydroxymethylgriseofulvin, respectively (Chart 1). This result clearly indicated that the hydroxymethyl groups are equatorial in both isomers (**4a** and **5a**). The structure of the most polar product (**7a**) was determined to be 5′α-hydroxymethylgriseofulvol by ^{1}H-NMR and MS analyses. On the other hand, **7a** was prepared from 5′α-hydroxymethylgriseofulvin (**4a**) by

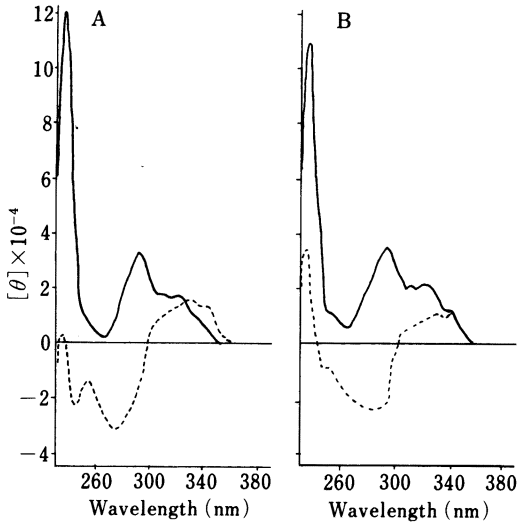


Fig. 1. CD Spectra A, **4a** (——) and **5a** (- - - -) in $CHCl_3$; B, **2** (——) and **6** (- - - -) in $CHCl_3$

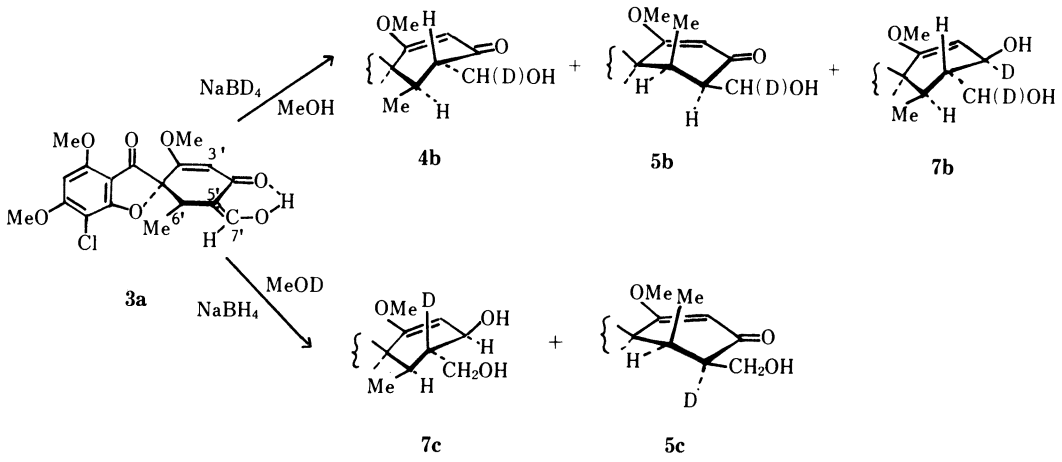


Chart 2

further reduction with $NaBH_4$, but treatment of 5′β-hydroxymethylgriseofulvin (**5a**) with $NaBH_4$ did not afford any reduction product, indicating that **5a** is sterically hindered in relation to hydride attack.

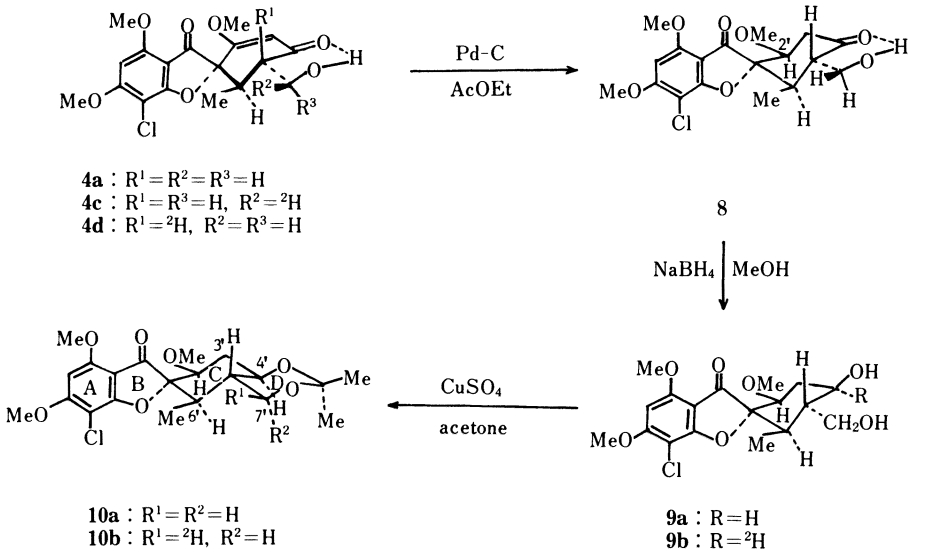
On the other hand, 5′-formylgriseofulvin (**3a**) was treated with deuterated sodium borohydride ($NaBD_4$) instead of unlabeled sodium borohydride in methyl alcohol, and with $NaBH_4$ and methyl alcohol-d_1 (Chart 2). The reduction products (**4b**, **5b**, **5c**, **7b**, and **7c**) in the above reactions were analyzed by MS and ^{1}H-NMR in order to determine the position of the incorporated deuterium atom. Thus, the products (**4b**, **5b**, and **7b**) of $NaBD_4$ treatment in methyl alcohol were determined to be deuterated at either one of the 7′-methylene protons nonstereospecifically. The other products (**5c** and **7c**) obtained by treatment with $NaBH_4$ in methyl alcohol-d_1 were proved to be 5′-deuterated products, [5′β-^{2}H]5′α-hydroxymethylgriseofulvol (**7c**) and [5′α-^{2}H]5′β-hydroxymethylgriseofulvin (**5c**). The reduction of **3a** with sodium borohydride or sodium borodeuteride presumably proceeds through a mechanism of 1,4-addition[5] giving the corresponding 5′-hydroxymethyl derivatives.

Since it was suggested[6] that the reduction with sodium borohydride in hydroxylic

solvents involves alkoxyborohydride, the hydroxyl hydrogen in methyl alcohol (or deuterium in methyl alcohol-d_1) will provide the proton (or deuteron) for completion of the reaction before aqueous work-up. This assumption can explain the results of the present studies which afforded the products (**5c** and **7c**) deuterated at the 5′-position.

Transformation of [7′-^{2}H]5′-Formylgriseofulvin to [7′-^{2}H]5′α-Hydroxymethylgriseofulvin by a Cell-Free System

In order to clarify the stereochemistry of hydrogenation of **3a** [7′-^{2}H]5′-formylgriseofulvin (**3b**) was prepared as a substrate, and the reaction was performed under the same conditions as used to obtain **3a** from **2** except for the use of deuterated methylformate (DCO_2CH_3) instead of unlabeled methylformate. The deuterium enrichment at the 7′-position of **3b** was >98% along with 28% at the 3′-position as determined by ^{1}H-NMR spectrum analysis. Evidence that the 7′-hydrogen atom of 5′-formylgriseofulvin (**3a**) is retained without being exchanged with hydrogen originating from water in the medium and also the stereochemistry of hydrogenation of **3a** were provided by analysis of the incubation product of **3b** with a cell-free system, which afforded a product (**4c**), whose ^{1}H-NMR



4a : $R^1=R^2=R^3=H$
4c : $R^1=R^3=H$, $R^2=^2H$
4d : $R^1=^2H$, $R^2=R^3=H$

8

10a : $R^1=R^2=H$
10b : $R^1=^2H$, $R^2=H$

9a : R=H
9b : R=^{2}H

Chart 3

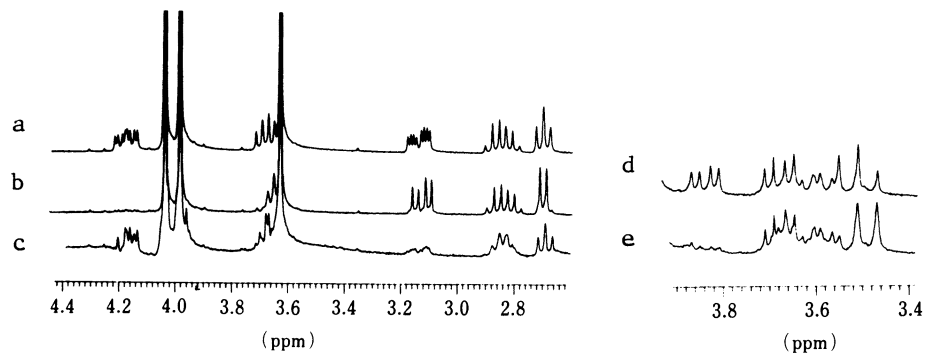


Fig. 2. Partial 270 MHz ^{1}H-NMR Spectra of **4a**, **10a**, **10b**, and the Enzymatic Transformation Products (**4c** and **4d**)

a, **4a**; b, **4c**; c, **4d**; d, **10a**; e, **10b**.

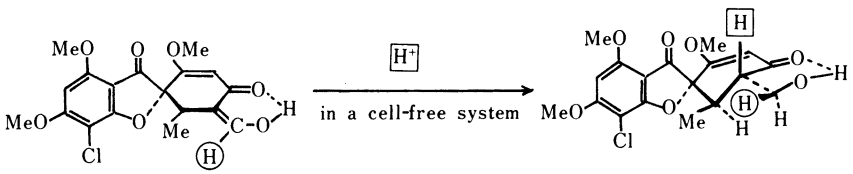
spectrum differed from that of **4a** in regard to the disappearance of the signal at 4.17 ppm and the change of the signal pattern at 3.68 ppm of **4a** from a doublet of doublets to a doublet at 3.66 ppm, indicating an isotopic effect (0.02 ppm) on the chemical shift.

To elucidate the stereochemistry of the deuteration of the enzymatic transformation product, the synthesis of the acetonide (**10b**), in which the conformation of the D ring is fixed, was carried out (Chart 3). In the preliminary experiment, **4a** was treated as shown in Chart 3 to give **10a**. Namely, **4a** was catalytically reduced to afford the 2′β-methoxy derivative (**8**) followed by $NaBH_4$ reduction to give **9a**. On the other hand, the 4′-proton signal of **9a** was assigned at 3.76 ppm, since in the ^{1}H-NMR spectrum of **9b** obtained from $NaBD_4$ reduction of **8** no signal at 3.76 ppm was observed. Moreover, the configuration of the 4′-OH group of **9a** was determined to be β, because the coupling constants of 4′-H with 3′α-, 3′β-, and 5′β-H were 5.9, 9.9, and 9.9 Hz, respectively. Next, **9a** was transformed to the acetonide (**10a**) which has a *trans*-junction in the C and D rings. In the ^{1}H-NMR spectrum, the chemical shifts of 7′α-H (axial) and 7′β-H (equatorial) of **10a** were unequivocally determined as 3.50 and 3.84 ppm, respectively, by analysis of the coupling constants. This conclusion is consistent with previous results which indicated that the equatorial proton of the methylene protons appears at lower field than the corresponding axial one.[7] Now, the enzymatic transformation product (**4c**) of **3b** was transformed to the acetonide (**10b**) under the same conditions as described above without purification of the corresponding derivatives of **8** and **9**. Comparison of the ^{1}H-NMR spectrum of **10b** with that of **10a** proved that the signal at 3.84 ppm (7′β-H) in **10a** disappears in **10b** and the 7′α-H signal (3.50 ppm) in **10a** is changed from a doublet of doublets to a doublet (3.49 ppm) due to deuteration at 7′β in **10b** (Fig. 2). This result clearly demonstrated that the enzymatic transformation of **3b** produces [7′*S*-^{2}H]5′α-hydroxymethylgriseofulvin (**4c**) stereospecifically.

Deuterium Incorporation into 5′α-Hydroxymethylgriseofulvin on Incubation of 5′-Formylgriseofulvin with a Cell-Free System Containing D_2O

Since the hydrogenation of 5′-formylgriseofulvin (**3a**) to 5′α-hydroxymethylgriseofulvin (**4a**) by a cell-free extract of *S. cinereocrocatus* stoichiometrically requires one proton and one hydride ion, 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 the two hydrogens on the hydrogenated product. The structure of the product was proved to be [5′β-^{2}H]5′α-hydroxymethylgriseofulvin (**4d**) by ^{1}H-NMR analysis (Fig. 2).

The above results clearly indicated that the 5′β-hydrogen and 7′-*pro*-*S*-hydrogen of 5′α-hydroxymethylgriseofulvin originate from a proton in the medium and the hydrogen atom at the 7′-position of 5′-formylgriseofulvin, respectively (Chart 4). The present results, together with the results on dehydrogriseofulvin and its derivatives previously described, demonstrate that the micro-organism, *Streptomyces cinereocrocatus* NRRL 3443, has an enzymatic activity which reduces the α-hydroxymethylene-ketone to α-hydroxymethyl-ketone.



3a 4a

Chart 4

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 and are reported as parts per million downfield from Me_4Si ($\delta=0$). Abbreviations used: s = singlet, d = doublet, br = broad, m = multiplet, dd = doublet of doublets, ddd = doublet of doublets of doublets, q = quartet. MS were recorded on a JEOL D-100 spectrometer at 75 eV ionizing potential. The optical rotations were measured on a JASCO DIP-140 digital 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. UV spectra were recorded on a JEOL UVIDEC 430B double-beam spectrophotometer. 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. Column chromatography was performed with Kanto Kagaku silica gel (100 mesh). The plates (precoated thin layer chromatography (TLC) plates, Silica gel 60F-254, Merck) were developed in benzene–acetone (7 : 3, v/v). The compounds were visualized under UV light and/or by spraying with concentrated H_2SO_4 and heating on an electric heater. High performance liquid chromatography (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. pH values were recorded on a LAB-O-MATE (Beckman-Toshiba, Ltd.).

Materials——NADH and NADPH were purchased from Boehringer Mannheim GmbH. Deuterium oxide (purity, 99.8 atom% D) was purchased from Merck Sharp & Dohme Canada Ltd., Montreal, Canada. Methyl alcohol-d_1 (purity, 99.5 atom% D) was purchased from Wako Pure Chemical Industries Ltd. All other reagents were purchased from commercial sources and were of analytical grade.

Preparation of the Cell-Free System and a Partially Purified Enzyme System——The preparation of the cell-free system of *S. cinereocrocatus* was undertaken as described in the previous report.[3e]

Determination of Protein——Protein concentration of the cell-free extract was determined by the method of Lowry *et al.*[8] with bovine serum albumin as a standard.

Syntheses of 5′-Formylgriseofulvin (3a) and [7′-^{2}H]5′-Formylgriseofulvin (3b)——5′-Formylgriseofulvin (**3a**) was synthesized by the method of Newman and Fields.[4] Recrystallization from benzene afforded **3a** as pale yellow needles, mp 196—197 °C (lit., 192 °C). MS: *m/z* 380 (M^+) (for the ^{35}Cl-compound), 214, 78 (base peak). ^{1}H-NMR δ (ppm): 1.04 (3H, d, J=6.8 Hz, 6′-CH_3), 3.42 (1H, dd, J=6.8, 1.8 Hz, 6′α-H), 3.66 (3H, s, 2′-OCH_3), 3.96 (3H, s, 4-OCH_3), 4.02 (3H, s, 6-OCH_3), 5.53 (1H, s, 3′-H), 6.12 (1H, s, 5-H), 7.10 (1H, dd, J=10.8, 1.8 Hz, =CHOH), 13.95 (1H, d, J=10.8 Hz, =CHOH). [7′-^{2}H]5′-Formylgriseofulvin (**3b**) was prepared under the same conditions used for **3a** from **2** except for the use of deuterated methylformate (DCO_2CH_3) instead of unlabeled methylformate. Recrystallization from benzene afforded **3b** as pale yellow needles, mp 204—205 °C. MS: $^2H_0$ 11%, $^2H_1$ 63%, $^2H_2$ 26%. ^{1}H-NMR δ (ppm): 1.04 (3H, d, J=6.6 Hz, 6′-CH_3), 3.42 (1H, q, J=6.6 Hz, 6′α-H), 3.66 (3H, s, 2′-OCH_3), 3.96 (3H, s, 4-OCH_3), 4.02 (3H, s, 6-OCH_3), 5.53 (0.6H, s, 3′-H), 6.12 (1H, s, 5-H), 13.96 (1H, br s, =CHOH).

$NaBH_4$ Reduction of 5′-Formylgriseofulvin (3a)——A solution of 5′-formylgriseofulvin (**3a**) (1 g, 2.6 mmol) and $NaBH_4$ (150 mg, 4.0 mmol) in MeOH (60 ml) was stirred for 100 min at room temperature. The reaction mixture was diluted with ice-H_2O, adjusted to pH 7 with 2 N HCl and then extracted with $CHCl_3$ (100 ml × 3). The chloroform solution was washed with water, dried (Na_2SO_4) and concentrated *in vacuo* to give a yellow oil (985 mg). The ratio of the starting material and the three products (**4a**, **5a**, and **7a**) in the reaction mixture was 79 : 8 : 8 : 5 by ^{1}H-NMR analysis. The mixture in benzene was chromatographed on silica gel (50 g). Elution with benzene–methylene chloride (50 : 50) and recrystallization from benzene gave the starting material as pale yellow needles, mp 196—197 °C. Elution with benzene–methylene chloride (10 : 90) and repeated recrystallization from acetone gave 5′α-hydroxymethylgriseofulvin (**4a**) as colorless needles, mp 211—212 °C, $[\alpha]_D^{20}$ +313.9° (c=0.69, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{19}ClO_7$: C, 56.47; H, 5.00. Found: C, 56.59; H, 5.01. MS: *m/z* 382 (M^+) (for the ^{35}Cl-compound), 364, 351, 310 (base peak), 225. ^{1}H-NMR δ (ppm): 0.99 (3H, d, J=6.6 Hz, 6′-CH_3), 2.73 (1H, dd, J=7.1, 5.8 Hz, 7′-OH), 2.84 (1H, dq, J=12.6, 6.6 Hz, 6′α-H), 3.13 (1H, ddd, J=12.7, 5.5, 3.0 Hz, 5′β-H), 3.63 (3H, s, 2′-OCH_3), 3.68 (1H, ddd, J=11.5, 5.8, 5.6 Hz, 7′-*pro-R*-H), 3.98 (3H, s, 4-OCH_3), 4.04 (3H, s, 6-OCH_3), 4.17 (1H, ddd, J=11.5, 7.1, 3.0 Hz, 7′-*pro-S*-H), 5.56 (1H, s, 3′-H), 6.13 (1H, s, 5-H). UV $\lambda_{max}^{CHCl_3}$ nm (ε): 242 (18770), 292 (21650), 325 (4870). CD (c=0.60, $CHCl_3$) $[\theta]^{25}$ (nm): +120950 (236) (positive maximum), +1270 (266), +33100 (291), +17820 (304). Repeated recrystallization of the material from the mother liquid from methanol gave 5′β-hydroxymethylgriseofulvin (**5a**) as colorless needles, mp 154—155 °C, $[\alpha]_D^{20}$ −86.0° (c=1.21, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{19}ClO_7$: C, 56.47; H, 5.00. Found: C, 56.53; H, 5.17. MS: *m/z* 382 (M^+) (for the ^{35}Cl-compound), 364, 351, 310, 215 (base peak). ^{1}H-NMR δ (ppm): 1.11 (3H, d, J=7.2 Hz, 6′-CH_3), 2.49 (1H, dq, J=7.2, 4.6 Hz, 6′α-H), 3.13 (1H, dd, J=9.9, 3.3 Hz, 7′-OH), 3.24 (1H, ddd, J=8.0, 4.6, 4.6 Hz, 5′α-H), 3.62 (1H, ddd, J=11.7, 9.9, 4.6 Hz, 7′-H), 3.68 (3H, s, 2′-OCH_3), 3.99 (3H, s, 4-OCH_3), 4.02 (3H, s, 6-OCH_3), 4.14 (1H, ddd, J=11.7, 7.9, 3.3 Hz, 7′-H), 5.61 (1H, s, 3′-H), 6.13 (1H, s, 5-H). UV: $\lambda_{max}^{CHCl_3}$ nm (ε): 242 (17240), 292 (17670), 322 (4390). CD (c=0.58, $CHCl_3$) $[\theta]^{25}$ (nm): +2660 (235), −22590 (244), −14610 (253), −30560 (275) (negative maximum), +15940 (328) (positive maximum). Elution with methylene chloride and recrystallization from

acetone gave 5′α-hydroxymethylgriseofulvol (**7a**), mp 213—214 °C. *Anal.* Calcd for $C_{18}H_{21}ClO_7$: C, 56.18; H, 5.50. Found: C, 56.30; H, 5.51. MS: *m/z* 384 (M^+) (for the ^{35}Cl-compound), 353, 215 (base peak), 112. ^{1}H-NMR δ (ppm): 0.83 (3H, d, *J*=6.2 Hz, 6′-CH_3), 2.29 (1H, m, 7′-OH), 2.40 (2H, m, 5′β- and 6′α-H), 2.62 (1H, d, *J*=8.3 Hz, 4′β-OH), 3.45 (3H, s, 2′-OCH_3), 3.69 (1H, ddd, *J*=10.8, 5.9, 4.5 Hz, 7′-H), 3.96 (3H, s, 4-OCH_3), 4.01 (3H, s, 6-OCH_3), 4.07 (1H, ddd, *J*=10.8, 5.9, 2.6 Hz, 7′-H), 4.45 (1H, ddd, *J*=8.3, 8.1, 2.3 Hz, 4′α-H), 5.06 (1H, d, *J*=2.3 Hz, 3′-H), 6.08 (1H, s, 5-H).

Reduction of 5′α-Hydroxymethylgriseofulvin (4a) with $NaBH_4$——A solution of 5′α-hydroxymethylgriseofulvin (**4a**) (49 mg, 0.13 mmol) and $NaBH_4$ (13 mg, 0.3 mmol) in MeOH (4 ml) was stirred for 100 min at room temperature. The reaction mixture was treated as described above. The residue (53 mg) in methylene chloride was chromatographed on silica gel (15 g). Elution with methylene chloride–methanol (99 : 1) and recrystallization of the product from acetone gave 44 mg of 5′α-hydroxymethylgriseofulvol (**7a**) (yield, 85%) as colorless needles, mp 213—214 °C. The MS and ^{1}H-NMR were identical with those of the standard compound (**7a**).

Attempted Reduction of 5′β-Hydroxymethylgriseofulvin (5a) with $NaBH_4$——1) A solution of 5′β-hydroxymethylgriseofulvin (**5a**) (49 mg, 0.13 mmol) and $NaBH_4$ (13 mg, 0.3 mmol) in MeOH (6 ml) was stirred for 100 min at room temperature. The reaction mixture was treated as described above, and the starting material was recovered unchanged (100%). 2) A solution of 5′β-hydroxymethylgriseofulvin (**5a**) (40 mg, 0.1 mmol) and $NaBH_4$ (40 mg, 1.0 mmol) in MeOH (6 ml) was stirred for 2 h at room temperature. The reaction mixture was treated as described above, and the starting material was recovered unchanged (98%).

Reduction of 5′-Formylgriseofulvin (3a) with $NaBD_4$——A solution of 5′-formylgriseofulvin (**3a**) (1.0 g, 2.6 mmol) and $NaBD_4$ (166 mg, 4.0 mmol) in MeOH (60 ml) was stirred for 100 min at room temperature. The reaction mixture was treated as described above. The ratio (67 : 11 : 7 : 14) of the starting material and the three products (**4b**, **5b** and **7b**) in the reaction mixture was determined by ^{1}H-NMR analyses. The product mixture (1.1 g) in benzene was chromatographed on silica gel (60 g). Elution with benzene–methylene chloride (40 : 60) and recrystallization from benzene gave the starting material as pale yellow needles, mp 196—197 °C. Elution with methylene chloride and repeated recrystallization from acetone gave [7′-^{2}H]5′α-hydroxymethylgriseofulvin (**4b**) as colorless needles, mp 211—212 °C. MS: $^2H_0$ 23%, $^2H_1$ 77%. ^{1}H-NMR δ (ppm): 0.99 (3H, d, *J*=6.6 Hz, 6′-CH_3), 2.67 (1H, m, 7′-OH), 2.85 (1H, m, 6′α-H), 3.13 (1H, dd, *J*=12.5, 3.0 Hz, 5′β-H), 3.64 (3H, s, 2′-OCH_3), 3.67 (0.6H, dd, *J*=11.1, 5.9 Hz, undeuterated 7′-*pro-R*-H), 3.98 (3H, s, 4-OCH_3), 4.03 (3H, s, 6-OCH_3), 4.16 (0.7H, dd, *J*=6.9, 3.0 Hz, undeuterated 7′-*pro-S*-H), 5.56 (1H, s, 3′-H), 6.13 (1H, s, 5-H). Repeated recrystallization of the material from the mother liquid from methanol gave [7′-^{2}H]5′β-hydroxymethylgriseofulvin (**5b**) as colorless needles, mp 154—155 °C. MS: $^2H_0$ 16%, $^2H_1$ 84%. ^{1}H-NMR δ (ppm): 1.11 (3H, d, *J*=7.2 Hz, 6′-CH_3), 2.49 (1H, dq, *J*=7.2, 4.6 Hz, 6′α-H), 3.12 (1H, br d, *J*=2.0 Hz, 7′-OH), 3.23 (1H, dd, *J*=7.6, 4.6 Hz, 5′α-H), 3.60 (0.6H, dd, *J*=10.4, 4.3 Hz, undeuterated 7′-H), 3.68 (3H, s, 2′-OCH_3), 3.99 (3H, s, 4-OCH_3), 4.02 (3H, s, 6-OCH_3), 4.13 (0.6H, dd, *J*=7.9, 3.3 Hz, undeuterated 7′-H), 5.61 (1H, s, 3′-H), 6.13 (1H, s, 5-H). Elution with methylene chloride–methanol (99 : 1) and recrystallization of the product from acetone gave [4′,7′-^{2}H]5′α-hydroxymethylgriseofulvol (**7b**) as colorless needles, mp 203—204 °C. MS: $^2H_0$ 2%, $^2H_1$ 16%, $^2H_2$ 82%. ^{1}H-NMR δ (ppm): 0.83 (3H, d, *J*=5.9 Hz, 6′-CH_3), 2.27 (1H, m, 7′-OH), 2.36 (2H, br s, 5′β- and 6′α-H), 2.56 (1H, br s, 4′β-OH), 3.45 (3H, s, 2′-OCH_3), 3.68 (0.5H, br dd, *J*=10.6, 9.2 Hz, undeuterated 7′-H), 3.96 (3H, s, 4-OCH_3), 4.01 (3H, s, 6-OCH_3), 4.05 (0.6H, br d, *J*=3.6 Hz, undeuterated 7′-H), 5.04 (1H, s, 3′-H), 6.08 (1H, s, 5-H).

Reduction of 5′-Formylgriseofulvin (3a) with $NaBH_4$ in MeOD——A solution of 5′-formylgriseofulvin (**3a**) (1.0 g, 2.6 mmol) and $NaBH_4$ (150 mg, 4.0 mmol) in MeOD (60 ml) was stirred for 100 min at room temperature. The reaction mixture was treated as described above. The ratio (55 : 21 : 1 : 23) of the starting material and the products (**5c** and **7c**) in the reaction mixture was determined by ^{1}H-NMR. Elution with benzene–methylene chloride (40 : 60) and recrystallization of the product from benzene gave the starting material as pale yellow needles, mp 196—197 °C. Elution with methylene chloride and recrystallization of the product from methanol gave [5′α-^{2}H]5′β-hydroxymethylgriseofulvin (**5c**) as colorless needles, mp 150—151 °C. MS: $^2H_0$ 13%, $^2H_1$ 87%. ^{1}H-NMR δ (ppm): 1.11 (3H, d, *J*=7.0 Hz, 6′-CH_3), 2.49 (1H, q, *J*=7.2 Hz, 6′α-H), 3.12 (1H, dd, *J*=10.2, 3.3 Hz, 7′-OH), 3.24 (0.1H, m, undeuterated 5′α-H), 3.62 (1H, dd, *J*=11.6, 10.9 Hz, 7′-H), 3.68 (3H, s, 2′-OCH_3), 3.99 (3H, s, 4-OCH_3), 4.02 (3H, s, 6-OCH_3), 4.14 (1H, dd, *J*=11.4, 3.3 Hz, 7′-H), 5.61 (1H, s, 3′-H), 6.13 (1H, s, 5-H). Elution with methylene chloride–methanol (99 : 1) and recrystallization of the product from acetone gave [5′β-^{2}H]5′α-hydroxymethylgriseofulvol (**7c**) as colorless needles, mp 212—213 °C. MS: $^2H_0$ 9%, $^2H_1$ 91%. ^{1}H-NMR δ (ppm): 0.83 (3H, d, *J*=6.6 Hz, 6′-CH_3), 2.30—2.40 (2.1H, m, 7′-OH, 6′α-H and undeuterated 5′β-H), 2.66 (1H, d, *J*=7.9 Hz, 4′β-OH), 3.45 (3H, s, 2′-OCH_3), 3.69 (1H, dd, *J*=10.7, 4.0 Hz, 7′-H), 3.96 (3H, s, 4-OCH_3), 4.01 (3H, s, 4-OCH_3), 4.06 (1H, dd, *J*=10.7, 5.9 Hz, 7′-H), 4.45 (1H, dd, *J*=7.9, 2.3 Hz, 4′α-H), 5.06 (1H, d, *J*=2.3 Hz, 3′-H), 6.08 (1H, s, 5-H).

Synthesis of the Acetonide (10a)——A suspension of 5% palladium–charcoal catalyst (150 mg) in ethyl acetate–MeOH (4 : 1, 30 ml) containing 5′α-hydroxymethylgriseofulvin (**4a**) (260 mg) was shaken under a stream of hydrogen at atmospheric pressure and at room temperature. The hydrogenation was stopped after 90 min. The catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. The residue (273 mg) was chromatographed on silica gel. Elution with benzene–methylene chloride (40 : 60) and recrystallization of the product from benzene gave colorless needles of 5′α-hydroxymethyl-2′,3′-dihydrogriseofulvin (**8**), mp 195—196 °C. MS: *m/z* 384 (M^+) (for the

^{35}Cl-compound), 293, 255 (base peak), 130, 85. ^{1}H-NMR δ (ppm): 0.94 (3H, d, J=6.6 Hz, 6′-CH_3), 2.29 (1H, dd, J=8.3, 6.3 Hz, 7′-OH), 2.43 (1H, dq, J=12.9, 6.6 Hz, 6′α-H), 2.81 (1H, dd, J=13.2, 5.6 Hz, 3′α-H), 3.23 (1H, br ddd, J=12.9, 6.1, 4.6 Hz, 5′β-H), 3.29 (3H, s, 2′β-OCH_3), 3.37 (1H, dd, J=13.0, 12.9 Hz, 3′β-H), 3.58 (1H, ddd, J=11.9, 8.3, 4.6 Hz, 7′-H), 3.84 (1H, dd, J=12.2, 5.6 Hz, 2′α-H), 3.98 (3H, s, 4-OCH_3), 4.03 (3H, s, 6-OCH_3), 4.08 (1H, ddd, J=12.0, 6.1, 2.3 Hz, 7′-H), 6.11 (1H, s, 5-H). A methanol solution (2 ml) of 5′α-hydroxymethyl-2′,3′-dihydrogriseofulvin (**8**) (83 mg) was treated with $NaBH_4$ (20 mg) at room temperature for 2 h. The reaction mixture was poured into water and adjusted to pH 7.0 with 0.5 N HCl. The reaction mixture was repeatedly extracted with chloroform and the extract was washed with water, dried (Na_2SO_4), and concentrated *in vacuo* (yield, 88 mg). The crude product was chromatographed on silica gel. Elution with methylene chloride–methanol (97 : 3) and recrystallization of the product from methanol gave colorless needles of 5′α-hydroxymethyl-2′,3′-dihydrogriseofulvol (**9a**, 15 mg), mp 131—132 °C. MS: m/z 386 (M^+) (for the ^{35}Cl-compound), 350, 321, 255 (base peak), 115, 58. ^{1}H-NMR δ (ppm): 0.81 (3H, d, J=6.6 Hz, 6′-CH_3), 1.97 (1H, dq, J=12.9, 6.6 Hz, 6′α-H), 2.23—2.37 (4H, m, 3′α-,3′β- and 5′β-H and 7′-OH), 2.76 (1H, br s, 4′-OH), 3.60—3.66 (2H, m, 2′α- and 7′-H), 3.27 (3H, s, 2′β-OCH_3), 3.76 (1H, br ddd, J=9.9, 9.9, 5.9 Hz, 4′α-H), 3.94 (3H, s, 4-OCH_3), 4.00 (3H, s, 6-OCH_3), 4.07 (1H, br d, J=10.6 Hz, 7′-H), 6.06 (1H, s, 5-H). An acetone solution (2 ml) of 5′α-hydroxymethyl-2′,3′-dihydrogriseofulvol (**9a**, 15 mg) was treated with anhydrous cupric sulfate at room temperature for 1 h. The cupric sulfate was removed by filtration and the filtrate was concentrated *in vacuo* to afford the acetonide (**10a**, 13 mg). MS: m/z 426 (M^+) (for the ^{35}Cl-compound), 411, 350, 255, 114 (base peak). ^{1}H-NMR δ (ppm): 0.72 (3H, d, J=6.9 Hz, 6′-CH_3), 1.46 (6H, d, J=7.9 Hz, 8′-CH_3), 1.80 (1H, dq, J=12.9, 6.9 Hz, 6′α-H), 2.15—2.48 (3H, m, 3′α-, 3′β- and 5′β-H), 3.25 (3H, s, 2′-OCH_3), 3.50 (1H, dd, J=11.2, 11.2 Hz, 7′α-H), 3.60 (1H, dd, J=10.6, 6.9 Hz, 4′α-H), 3.67 (1H, dd, J=11.9, 5.2 Hz, 2′α-H), 3.84 (1H, dd, J=11.2, 5.0 Hz, 7′β-H), 3.94 (3H, s, 4-OCH_3), 3.99 (3H, s, 6-OCH_3), 6.06 (1H, s, 5-H).

Synthesis of [4′α-^{2}H]5′α-Hydroxymethyl-2′,3′-dihydrogriseofulvol (9b)——A methanol solution (2 ml) of 5′α-hydroxymethyl-2′,3′-dihydrogriseofulvin (85 mg) was treated with $NaBD_4$ (20 mg) at room temperature for 2 h. The reaction mixture was worked up as described above. Recrystallization from methanol afforded [4′α-^{2}H]5′α-hydroxymethyl-2′,3′-dihydrogriseofulvol (**9b**) as colorless needles, mp 131—132 °C. MS: $^2H_0$ 2%, $^2H_1$ 98%. ^{1}H-NMR δ (ppm): 0.81 (3H, d, J=6.6 Hz, 6′-CH_3), 1.97 (1H, dq, J=12.9, 6.0 Hz, 6′α-H), 2.23—2.37 (4H, m, 3′α-, 3′β- and 5′β-H, and 7′-OH), 2.76 (1H, br s, 4′-OH), 3.60—3.66 (2H, m, 2′α- and 7′-H), 3.94 (3H, s, 4-OCH_3), 4.00 (3H, s, 6-OCH_3), 4.07 (1H, br d, J=10.6 Hz, 7′-H), 6.06 (1H, s, 5-H).

Transformation of 5′-Formylgriseofulvin (3a) to 5′α-Hydroxymethylgriseofulvin (4a) by a Cell-Free System——The incubation and separation were carried out essentially as described in the previous paper[3d] except that 50 mg of 5′-formylgriseofulvin (**3a**) was used as a substrate and 100 mg of NADPH was added to the cell-free system (500 ml). The residue from the incubation mixture consisted of 5′α-hydroxymethylgriseofulvin (82% yield, by ^{1}H-NMR analysis). Column chromatography of the residue on silica gel (30 g) and recrystallization from acetone gave 5′α-hydroxymethylgriseofulvin (**4a**), mp 211—212 °C. ^{1}H-NMR, MS, UV and CD data were identical with those of a standard sample (**4a**).

Attempted Transformation of 3a with a Partially Purified Enzyme System——All experimental conditions were essentially the same as described in the previous paper.[3e] The starting material was recovered unchanged.

Transformation of [7′-^{2}H]5′-Formylgriseofulvin (3b) to [7′*S*-^{2}H]5′α-Hydroxymethylgriseofulvin (4c) by a Cell-Free System——The incubation and separation were carried out essentially as described in the previous papers[3] except that 60 mg of [7′-^{2}H]5′-formylgriseofulvin (**3b**) was used as a substrate in the cell-free system (300 ml). The residue from the incubation mixture consisted of 5′α-hydroxymethylgriseofulvin (80% yield, by ^{1}H-NMR analysis). Column chromatography of the residue on silica gel (30 g) and recrystallization of the product from acetone gave [7′*S*-^{2}H]5′α-hydroxymethylgriseofulvin (**4c**), mp 211—212 °C. MS: $^2H_0$ 8%, $^2H_1$ 65%, $^2H_2$ 27%. ^{1}H-NMR δ (ppm): 0.99 (3H, d, J=6.6 Hz, 6′-CH_3), 2.70 (1H, d, J=5.8 Hz, 7′-OH), 2.84 (1H, dq, J=12.6, 6.6 Hz, 6′α-H), 3.13 (1H, dd, J=12.6, 5.5 Hz, 5′β-H), 3.63 (3H, s, 2′-OCH_3), 3.66 (1H, d, J=5.9 Hz, 7′-*pro*-*R*-H), 3.98 (3H, s, 4′-OCH_3), 4.04 (3H, s, 6-OCH_3), 4.17 (0.1H, m, undeuterated 7′-*pro*-*S*-H), 5.56 (0.6H, s, 3′-H), 6.13 (1H, s, 5-H).

Preparation of [7′β-^{2}H]Acetonide (10b) from 4c——**4c** was transformed to the acetonide (**10b**) without purification of the intermediates. In the final step of the formation of [7′β-^{2}H]acetonide (**10b**), the product was purified by HPLC. MS: $^2H_0$ 8%, $^2H_1$ 66%, $^2H_2$ 26%. ^{1}H-NMR δ (ppm): 0.72 (3H, d, J=6.9 Hz, 6′-CH_3), 1.46 (6H, d, J=7.9 Hz, 8′-CH_3), 1.80 (1H, dq, J=12.9, 6.9 Hz, 6′α-H), 2.15—2.48 (2.6H, m, 3′α-, 3′β- and 5′β-H), 3.25 (3H, s, 2′-OCH_3), 3.50 (1H, dd, J=11.2, 11.2 Hz, 7′α-H), 3.60 (1H, dd, J=10.6, 6.9 Hz, 4′α-H), 3.67 (1H, ddd, J=11.9, 5.2, 5.2 Hz, 2′α-H), 3.84 (0.1H, m, undeuterated 7′β-H), 3.94 (3H, s, 4-OCH_3), 3.99 (3H, s, 6-OCH_3), 6.06 (1H, s, 5-H).

Transformation of 5′-Formylgriseofulvin (3a) into [5′β-^{2}H]5′α-Hydroxymethylgriseofulvin (4d) by a Cell-Free System Containing D_2O——All of the experiments were carried out essentially as described above except that 0.03 M phosphate buffer prepared with D_2O was used as a buffer, and 60 mg of 5′-formylgriseofulvin was added to the cell-free system (300 ml). The residue from the incubation mixture was found to consist of 5′α-hydroxymethylgriseofulvin (60% yield) by ^{1}H-NMR analysis. Column chromatography of the residue on silica gel (30 g) and recrystallization of the product from acetone gave [5′β-^{2}H]5′α-hydroxymethylgriseofulvin (**4d**), mp 211—212 °C. MS: $^2H_0$ 34%, $^2H_1$ 66%. ^{1}H-NMR δ (ppm): 0.99 (3H, d, J=6.6 Hz, 6′-CH_3), 2.73 (1H, dd, J=7.1, 5.8 Hz, 7′-OH), 2.84 (1H, br q, J=6.6 Hz, 6′α-H), 3.13 (0.3H, ddd, J=12.7, 5.4, 3.0 Hz, undeuterated 5′β-H), 3.63 (3H, s, 2′-OCH_3), 3.68 (1H, br dd, J=

11.5, 5.9 Hz, 7′-*pro*-*R*-H), 3.98 (3H, s, 4-OCH_3), 4.03 (3H, s, 6-OCH_3), 4.15 (1H, br dd, J=11.5, 7.1 Hz, 7′-*pro*-*S*-H), 5.56 (1H, s, 3′-H), 6.13 (1H, s, 5-H).

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