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A NOVEL BIOSYNTHETIC STUDY OF GRISEOFULVIN BY  $^2\text{H}$  NUCLEAR  
MAGNETIC RESONANCE: DETERMINATION OF DEUTERIUM  
INCORPORATION FROM  $[2\text{-}^2\text{H}_3]\text{-ACETATE}$  BY *PENICILLIUM URTICAE* \*

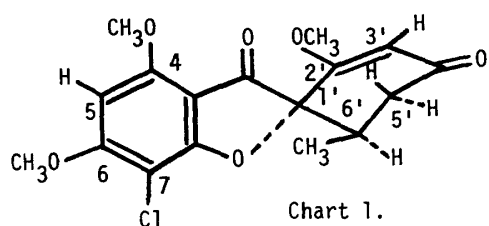
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In the elucidation of the biosynthetic pathways, the use of  $^{13}\text{C}$  nmr combined with  $^{13}\text{C}$ -label-precursors has been common practice to locate the enriched site and determine the skeleton-formation. This method, however, does not provide an unambiguous information on biosynthetic pathways involving hydrogen. For this purpose, the use of  $^2\text{H}$  nmr in case of  $^2\text{H}$ -labeled precursors seems to have potential utility for the location of deuterium incorporation, together with mass-spectrometric analysis. Although very few works have been done on this subject partly because of fear of lower sensitivity and wider line-width of deuterium signal, recent developments of the pulsed Fourier transform nmr method have enabled us to study various types of  $^2\text{H}$  nmr to chemical and biological problems. We now wish to demonstrate that direct evidence of deuterium incorporation and its stereochemical course on biosynthesis of griseofulvin are obtained from  $^2\text{H}$  nmr when  $[2\text{-}^2\text{H}_3]\text{-acetate}$  is used as a tracer for the biosynthesis, which is in good agreement with the previous studies using  $[2\text{-}^3\text{H}, ^{14}\text{C}]\text{-acetate}$ .

$^2\text{H}$  nmr spectra were recorded by a JEOL PFT-100/EC-100 pulsed Fourier transform spectrometer operating at 15.28 MHz with proton-noise decoupling. All samples of chloroform solution were contained in 10 mm o. d. sample tubes. Field-frequency control was performed on the internal signal of  $\text{C}_6\text{F}_6$ , which was added by amounts of a few drops in the chloroform solution. The biosynthetically deuterated griseofulvin (**1a**) was prepared from sodium  $[2\text{-}^2\text{H}_3]\text{-acetate}$  by *Penicillium urticae* as previously reported. In order to perform unambiguous assignment of  $^2\text{H}$  signals, a series of selectively deuterated griseofulvin samples were prepared (Chart 1). In Figure 1A is shown a  $^2\text{H}$  nmr spectrum of biosynthetically deuterated griseofulvin (**1a**) in  $\text{CHCl}_3$  solution (4 w/%). The lowermost sharp signal arises from  $\text{CDCl}_3$  occurring in  $\text{CHCl}_3$  of natural abundance (0.02%). The assignment of  $^2\text{H}$  nmr signals is straightforward to that of  $^1\text{H}$  nmr, since chemical-shift displacement due to isotope effect are usually negligible. In this communication, however, the peak-assignments were made with the aid of  $^2\text{H}$  signals of selectively deuterated griseofulvin samples described above. First, the peaks of  $2'\text{-OCH}_2\text{D}$  and  $5\text{-D}$  are assigned by comparing  $^2\text{H}$  nmr spectrum of **1a** with that of **1b** (Figure 1B), in which deuteriums are removed

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- $\underline{1a}$ ; biosynthetically deuterated  
 $\underline{1b}$ ; removed the deuteriums at 2' and 3' positions of  $\underline{1a}$   
 $\underline{1c}$ ; 5' $\alpha$ , 5' $\beta$ -D<sub>2</sub>  
 $\underline{1d}$ ; 2'-OCHD<sub>2</sub>  
 $\underline{1e}$ ; 2'-OCH<sub>2</sub>D, 3'-D

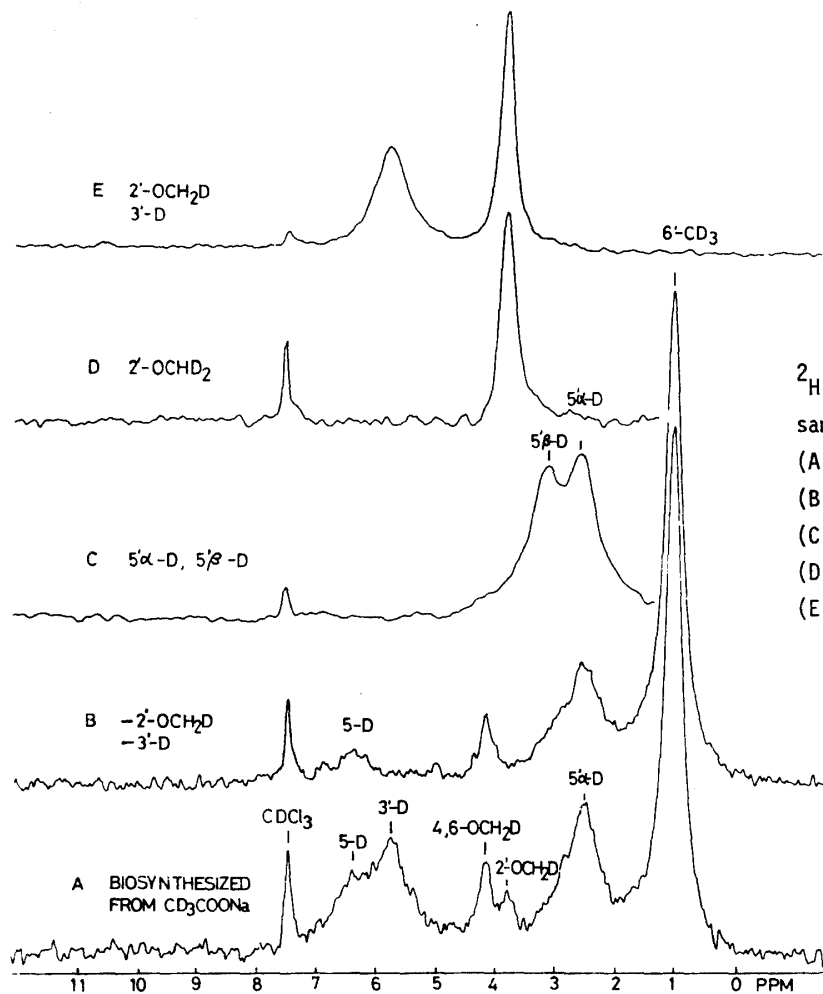


Figure 1.

- <sup>2</sup>H nmr spectra of griseofulvin samples in CHCl<sub>3</sub> solutions.  
 (A)  $\underline{1a}$ , 40 mg/ml, 1100 accum.  
 (B)  $\underline{1b}$ , 29 mg/ml, 502 accum.  
 (C)  $\underline{1c}$ , 35 mg/ml, 1000 accum.  
 (D)  $\underline{1d}$ , 16 mg/ml, 1000 accum.  
 (E)  $\underline{1e}$ , 27 mg/ml, 200 accum.

at 2'-methoxyl and 3'-position. The assignment of 2'-OCH<sub>2</sub>D signal is also confirmed by employing [2'-OCH<sub>2</sub>D, 3'-D]-griseofulvin ( $\underline{1e}$ , Figure 1E) and [2'-OCHD<sub>2</sub>]-griseofulvin ( $\underline{1d}$ , Figure 1D) as the reference samples. In comparison with <sup>2</sup>H nmr of  $\underline{1c}$  (Figure 1C), deuterium at 5'-position is confirmed to have been incorporated exclusively at  $\alpha$  configuration. This result is in agreement with the previous studies on [2-<sup>3</sup>H, <sup>14</sup>C]-acetate tracer. Further, <sup>2</sup>H T<sub>1</sub> values show that deuteriums incorporated at methyl or methoxyl groups where internal rotation will be allowed in addition to overall molecular tumbling are found to give large T<sub>1</sub> values (86, 106 and 104 msec for 6'-CD<sub>3</sub>, 2'-OCH<sub>2</sub>D and 4, 6-OCH<sub>2</sub>D, respectively) compared with 5' $\alpha$ -D and

(45 and 46 msec, respectively).

In contrast to the case of  $^{13}\text{C}$  nmr, nuclear Overhauser enhancement by proton-decoupling is negligible for  $^2\text{H}$  nuclei where the quadrupole relaxation mechanism is dominant. Accordingly, integrated peak-intensities are proportional to the extent of deuterium-incorporation by biosynthesis. The relative  $^2\text{H}$  peak-intensities of **1a** are: 44% ( $6'\text{-CD}_3$ ), 23% ( $5'\alpha\text{-D}$ ) 3.3% ( $2'\text{-OCH}_2\text{D}$ ), 6.3% ( $4,6\text{-OCH}_2\text{D}$ ) and 24% ( $3'\text{-D}$  and  $5\text{-D}$ ). The comparison of the peak-intensities between  $6'\text{-CD}_3$  and  $5'\alpha\text{-D}$  strongly suggests that  $6'$  position might be  $\text{CHD}_2$  instead of  $\text{CD}_3$ . This would be easily proved if doubling of the  $^2\text{H}$  signal due to *geminal*  $^2\text{H}\text{-}^1\text{H}$  spin coupling were observed. Unfortunately, no such a fine structure was observed in the proton-coupled  $^2\text{H}$  spectrum recorded under the condition of turning-off proton-decoupler. It is expected that this situation arises when peak-splittings due to  $^2\text{H}\text{-}^1\text{H}$  spin-couplings, (the splitting of which being  $1/6$  of corresponding  $^1\text{H}\text{-}^1\text{H}$  couplings) are buried within relatively broader line-width. Employing  $1/T_1$  as a theoretical limit of a line-width free from various broadening factors such as magnetic inhomogeneity and unresolved  $^2\text{H}\text{-}^1\text{H}$  spin-couplings, it is predicted that no fine structure could be observed unless otherwise  $\pi T_1 J_{\text{DH}} \gg 1$ . Here  $J_{\text{DH}}$  stands for  $^2\text{H}$  spin coupling constant. In our present case,  $\pi T_1 J_{\text{DH}} \sim 0.5$  is obtained from the values of  $T_1 \sim 100$  msec and  $J_{\text{DH}} \sim 1.7$  Hz. This value predicts that proton-decoupling experiment will alter spectral pattern to some extent. In fact, the peak heights of  $2'\text{-OCH}_2\text{D}$  and  $4,6\text{-OCH}_2\text{D}$  are found to be increased by amounts of 27% and 21%, respectively, when compared with those of proton-coupled spectrum. Therefore, the enhanced peak-height of  $6'$ -methyl by amount of 17% suggests that the deuterium-incorporation at  $6'$ -position is apparently like  $\text{CHD}_2$ . Further, it is of interest to note that 9.6% of deuterium is incorporated at unexpected methoxyl groups ( $4,6\text{-}$  and  $2'\text{-OCH}_2\text{D}$ ). Such an analysis could not readily be performed by other physical techniques.

In conclusion, it is proved that  $^2\text{H}$  nmr is very powerful nondestructive method to study biosynthetic pathways involving hydrogen.