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A NOVEL BIOSYNTHETIC STUDY OF GRISEOFULVIN BY ^2H NUCLEAR MAGNETIC RESONANCE: DETERMINATION OF DEUTERIUM INCORPORATION FROM [2-$^2H_3$]-ACETATE BY *PENICILLIUM URTICAE* *

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In the elucidation of the biosynthetic pathways, the use of ^{13}C nmr combined with ^{13}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 ^2H nmr in cass of ^2H-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 ^2H 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 ^2H nmr when [2-$^2H_3$]-acetate is used as a tracer for the biosynthesis, which is in good agreement with the previous studies using [2-^3H, ^{14}C]-acetate.

^2H 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 C_6F_6, which was added by amounts of a few drops in the chloroform solution. The biosynthetically deuterated griseofulvin (**1a**) was prepared from sodium [2-$^2H_3$]-acetate by *Penicillium urticae* as previously reported. In order to perform unambiguous assignment of ^2H signals, a series of selectively deuterated griseofulvin samples were prepared (Chart 1). In Figure 1A is shown a ^2H nmr spectrum of biosynthetically deuterated griseofulvin (**1a**) in $CHCl_3$ solution (4 w/%). The lowermost sharp signal arises from $CDCl_3$ occuring in $CHCl_3$ of natural abundance (0.02%). The assignment of ^2H nmr signals is straightforward to that of ^1H 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 ^2H signals of selectively deuterated griseofulvin samples described above. First, the peaks of 2′-OCH_2D and 5-D are assigned by comparing ^2H nmr spectrum of **1a** with that of **1b** (Figure 1B), in which deuteriums are removed

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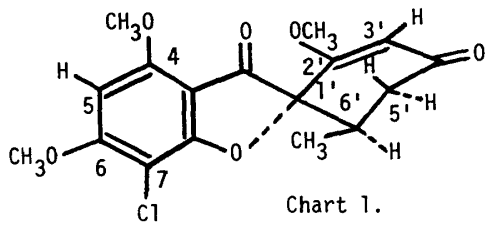

Chart 1.

1a; biosynthetically deuterated
1b; removed the deuteriums at 2' and 3' positions of 1a
1c; 5'α, 5'β-D_2
1d; 2'-$OCHD_2$
1e; 2'-OCH_2D, 3'-D

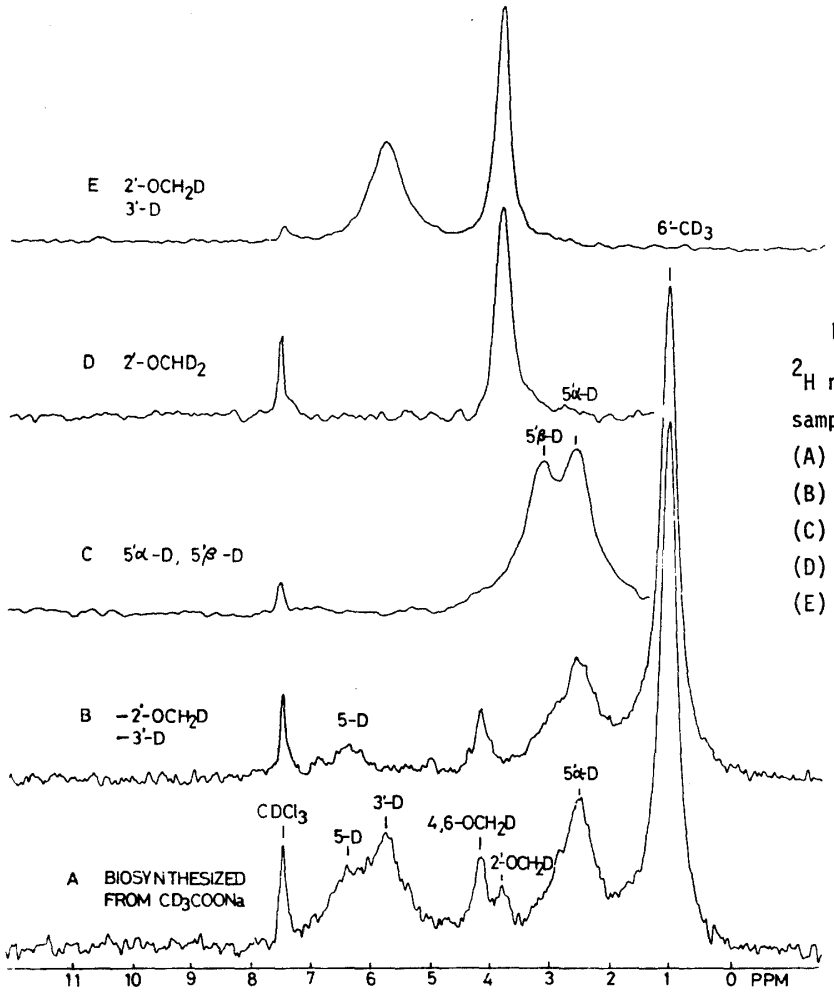

Figure 1.
^2H nmr spectra of griseofulvin samples in $CHCl_3$ solutions.
(A) 1a, 40 mg/ml,1100 accum.
(B) 1b, 29 mg/ml,502 accum.
(C) 1c, 35 mg/ml,1000 accum.
(D) 1d, 16 mg/ml,1000 accum.
(E) 1e, 27 mg/ml, 200 accum.

at 2'-methoxyl and 3'-position. The assignment of 2'-OCH_2D signal is also confirmed by employing [2'-OCH_2D, 3'-D]-griseofulvin (**1e**, Figure 1E) and [2'-$OCHD_2$]-griseofulvin (**1d**, Figure 1D) as the reference samples. In comparison with ^2H nmr of **1c** (Figure 1C), deuterium at 5'-position is confirmed to have been incorporated exclusively at α configuration. This result is in agreement with the previous studies on [2-^3H, ^{14}C]-acetate tracer. Further, ^2H T_1 values show that deuteriums incorporated at methyl or methoxyl groups where internal rotation will be allowed in addiion to overall molecular tumbling are found to give larges T_1 values (86, 106 and 104 msec for 6'-CD_3, 2'-OCH_2D and 4, 6-OCH_2D, respeetively) compared with 5'α-D and

(45 and 46 msec, respectively).

In contrast to the case of ^{13}C nmr, nuclear Overhauser enhancement by proton-decoupling is negligible for ^{2}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}H peak-intensities of **1a** are : 44% (6'-CD_3), 23% (5' α-D) 3.3% (2'-OCH_2D), 6.3% (4,6-OCH_2D) and 24% (3'-D and 5-D). The comparison of the peak-intensities between 6'-CD_3 and 5'α-D strongly suggests that 6' position might be CHD_2 instead of CD_3. This would be easily proved if doubling of the ^{2}H signal due to *geminal* ^{2}H-^{1}H spin coupling were observed. Unfortunately, no such a fine structure was observed in the proton-coupled ^{2}H spectrum recorded under the condition of turning-off proton-decoupler. It is expected that this situation arises when peak-splittings due to ^{2}H-^{1}H spin-couplings, (the splitting of which being 1/6 of corresponding ^{1}H-^{1}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}H-^{1}H spin-couplings, it is predicted that no fine structure could be observed unless otherwise $\pi T_1 J_{DH} \gg 1$. Here J_{DH} stands for ^{2}H spin coupling constant. In our present case, $\pi T_1 J_{DH} \sim 0.5$ is obtained from the values of $T_1 \sim 100$ mesc and $J_{DH} \sim 1.7$ Hz. This value predicts that proton-decoupling experiment will alter spectral pattern to some extent. In fact, the peak heights of 2'-OCH_2D and 4. 6-OCH_2D 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 CHD_2. Further, it is of interest to note that 9.6% of deuterium is incorporated at unexpected methoxyl groups (4,6- and 2'-OCH_2D). Such an analysis could not readily be performed by other physical techniques.

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