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A NOVEL BIOSYNTHETIC STUDY OF GRISEOFULVIN BY ^{2}H NUCLEAR MAGNETIC RESONANCE: DETERMINATION OF DEUTERIUM INCORPORATION FROM [2-$^{2}H_3$]-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-labeled precursors has been common practice to locate the enriched site and determine the skeleton-formation.[1] This method, however, does not provide an unambiguous information on biosynthetic pathways involving hydrogen. For this purpose, the use of ^{2}H nmr in case of ^{2}H-labeled precursors seems to have potential utility for the location of deuterium incorporation, together with mass-spectrometric analysis.[2] Although very few works have been done on this subject[3] 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}H nmr to chemical and biological problems.[4] We now wish to demonstrate that direct evidence of deuterium incorporation and its stereochemical course on biosynthesis of griseofulvin are obtained from ^{2}H nmr when [2-$^{2}H_3$]-acetate is used as a tracer for the biosynthesis, which is in good agreement with the previous studies using [2-^{3}H,^{14}C]-acetate.[5]

^{2}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 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-$^{2}H_3$]-acetate by Penicillium urticae as previously reported[2,5] (d_0 61.4, d_1 4.3, d_2 5.9, d_3 8.6, d_4 10.6, d_5 6.4 %, and d_7 and $d_8 < 1$ % by mass-spectrometry). In order to perform unambiguous assignment of ^{2}H signals, a series of selectively deuterated griseofulvin samples were prepared in the following ways (Chart 1). Griseofulvic acid[6] obtained by hydrolysis of 1a (250 mg) was submitted to methylation with CH_2N_2 in CH_3OH-ether to afford a mixture of 1b and deuterated isogriseofulvin, the former of which was separated by silica-gel column chromatography (80 mg) (d_0 56.7, d_1 6.9, d_2 11.6, d_3 14.8, d_4 6.6, d_5 2.8 and d_6 1 %). [5',5'-^{2}H]-Griseofulvin (1c) was prepared under essentially the same conditions reported[7] (d_0 21.5, d_1 53.6 and d_2 24.9 %). A solution of undeuterated griseofulvic acid in CH_3OH-ether was methylated with [^{2}H]-CH_2N_2[8] to afford 1d (d_0 68.8 d_1 24.8, and d_2 6.1 %). Finally, heating the griseofulvic acid in $CHCl_3$ containing D_2O for 1 hr followed by evaporation of solvent under reduced pressure gave a deuterated acid, which was subsequently methylated with CH_2N_2 in CH_3OD-ether to afford 1e (d_0 2.4, d_1 19.2, d_2 71.5, and d_3 5.0 %).

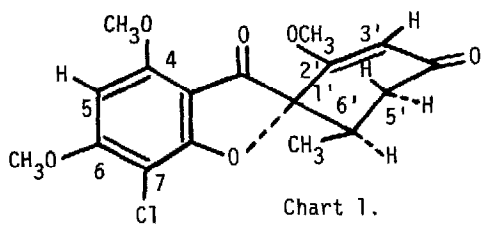

Chart 1.

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

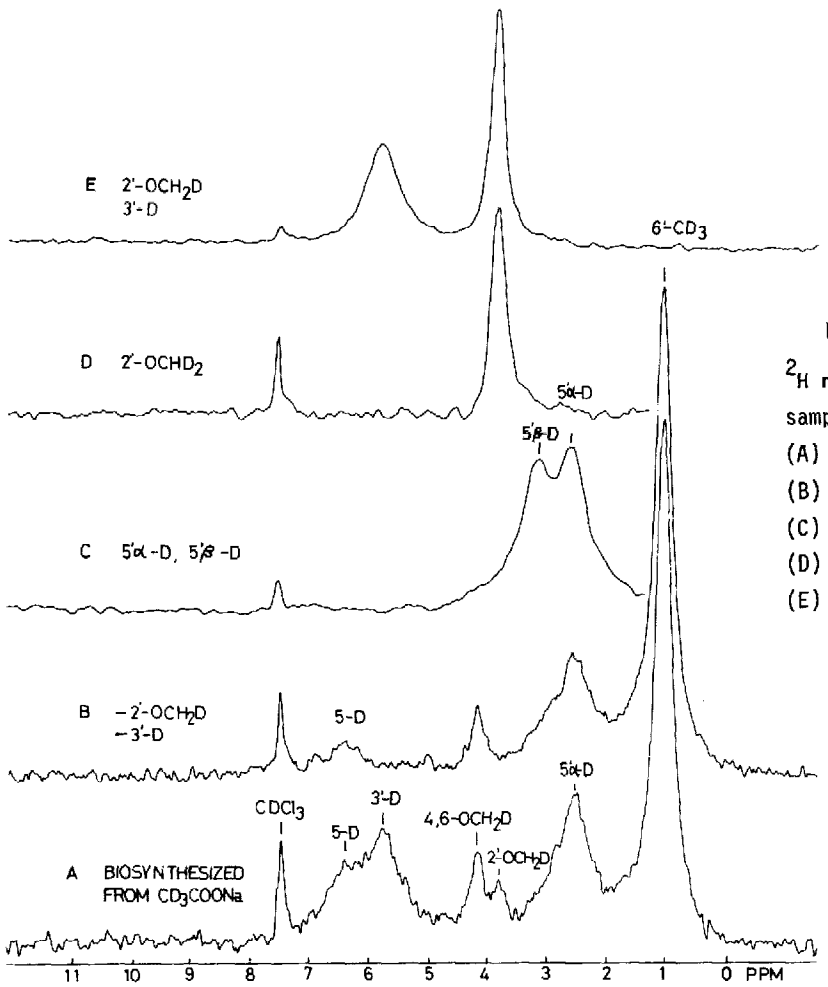

Figure 1.
^2H nmr spectra of griseofulvin samples in $CHCl_3$ solutions.
(A) 1a, 40 mg/ml,1100 accum.
(B) 1b, 29 mg/ml,502 accum.
(C) 1c, 35 mg/ml,1000 accum.
(D) 1d, 16 mg/ml,1000 accum.
(E) 1e, 27 mg/ml, 200 accum.

In Figure 1A is shown a ^2H nmr spectrum of biosynthetically deuterated griseofulvin (1a) in $CHCl_3$ solution (4 w/v %). 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 displacements 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 as-

signed by comparing ^2H nmr spectrum of 1a with that of 1b (Figure 1B), in which deuteriums are removed 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.[5] Further, ^2H T_1 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 larger T_1 values[9](86,106 and 104 msec for 6'-CD_3, 2'-OCH_2D and 4,6-OCH_2D, respectively) compared with 5'α-D and 3'-D (45 and 46 msec, respectively).

In contrast to the case of ^{13}C nmr, nuclear Overhauser enhancement by proton-decoupling is negligible for ^2H nuclei where the quadrupole relaxation mechanism is dominant. Accordingly, integrated peak-intensities are proportional to the extent of deuterium-incorporation by biosynthesis. The relative ^2H 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 ^2H signal due to geminal ^2H-^1H spin coupling were observed. Unfortunately, no such a fine structure was observed in the proton-coupled ^2H spectrum recorded under the condition of turning-off proton-decoupler (Figure 2A). It is expected that this situation arises when peak-splittings due to ^2H-^1H spin-couplings (the splitting of which being 1/6 of corresponding ^1H-^1H couplings) are buried within relatively broader line-width. Employing $1/\pi T_1$ as a theoretical limit of a line-width free from various broadening factors such as magnetic inhomogeneity and unresolved ^2H-^1H spin-couplings, it is predicted that no fine structure could be observed unless otherwise $\pi T_1 J_{DH} \gg 1$.[10] Here J_{DH} stands for ^2H spin coupling constant. In our

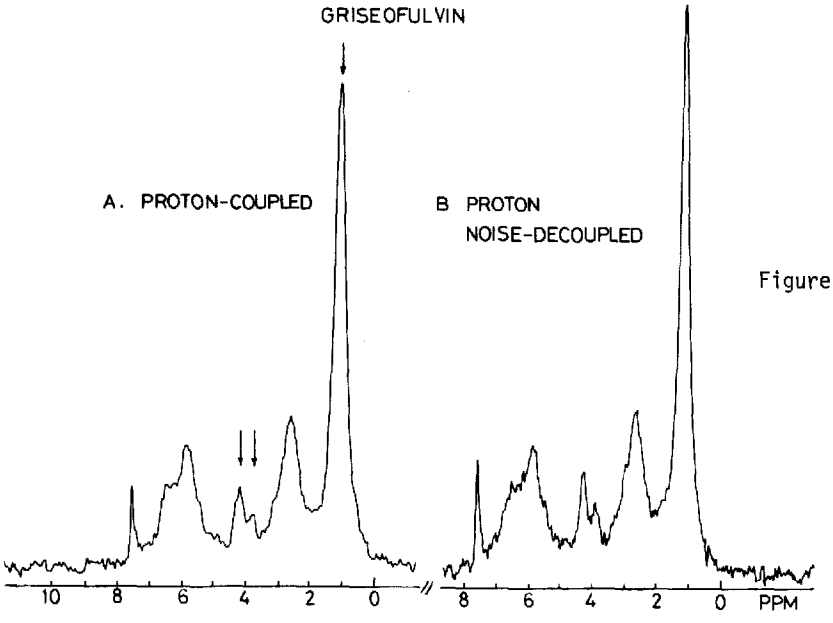

Figure 2. Comparison of proton-coupled and proton-decoupled ^2H nmr spectra. See changes of the peak-heights marked by the arrows.

present case, $\pi T_1 J_{DH} \sim 0.5$ is obtained from the values of $T_1 \sim 100$ msec 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 (Figure 2A and 2B). 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.[11] 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 ^2H nmr is very powerful nondestructive method to study biosynthetic pathways involving hydrogen.

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References and Notes

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(6) A sample of combined griseofulvin in several fermentations was used for the hydrolysis.

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(11) Data of mass-spectrometric analysis of fragment ion,* m/e 69 (CH_3-CH=CH-C≡O^+), together with the relative intensity of ^2H nmr at 5'α and 6'-methyl suggest that 6'-methyl deuterated consists of 75-89 % of 6'-CHD_2 and 14-22 % of 6'-CD_3 with a slight possibility for the presence of 6'-CH_2D. However, the mass data of griseofulvin sample obtained by short-time incubation suggest that 6'-methyl deuterated consists of CD_3 component only. *) J.A. Ballantine and R.G.Fenwich, Organic Mass Spectrometry, 2, 1145(1969)