A ^2H NMR STUDY ON BIOSYNTHESIS

Evidence of hydrogen exchange at the chain-starter methyl group of griseofulvin manifested by ^2H incorporation from deuterium oxide in medium and from sodium acetates

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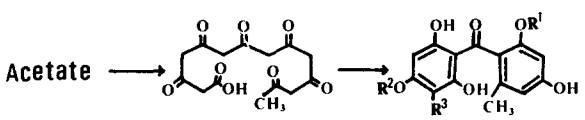
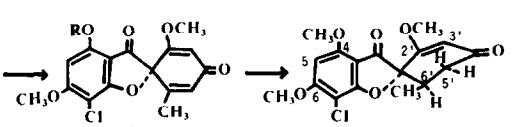
1. Introduction

The mechanism of heptaketide griseofulvin (**1**) biosynthesis has been examined by many workers [1–18] (see scheme 1). Still, the fate of hydrogen atoms of the chain-starter methyl group is not yet fully understood. For this purpose, ^2H NMR has proven to be a very powerful tool to examine the fate and stereochemical course of proton(s) during biosynthesis [17–25], even if the extent of deuterium incorporation is considerably low (~1%). In this connection, our previous ^2H NMR study on griseofulvin from [2-$^2H_3$]acetate [17] showed that 6′-methyl group of the chain-starter is apparently like CHD_2. In order to make the origin of hydrogen atom clear, we attempted to analyze ^2H NMR spectra of griseofulvin samples supplemented with: (a) sodium [2-^{13}C,2-$^2H_3$]acetate; (b) sodium acetate and deuterium oxide in medium; (c) sodium [2-^{13}C]acetate and deuterium oxide. Here we report evidence of participation of medium water into the methyl group of chain-starter, with use of various kinds of acetate precursors, as a result of hydrogen exchange.

Scheme 1



2 3



4 1

3a: $R^1 = R^2 = R^3 = H$. 3b: $R^1 = R^3 = H$, $R^2 = CH_3$.
3c: $R^3 = H$, $R^1 = R^2 = CH_3$. 3d: $R^1 = R^2 = CH_3$, $R^3 = Cl$.

4a: R = H. 4b: $R = CH_3$.

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2. Materials and methods

The biosynthetically deuterated griseofulvin samples were obtained in buffers replacing the original culture solution as follows. The mycelium obtained from 7-day-old shaken cultures of *Penicillium urticae* in medium 1 [18] was suspended in medium 2,3,4 or 5 for the biosynthetic experiments, respectively. The preparation of the standard [^2H]griseofulvin sample was described [17] in the culture in medium 2, which contained sodium [2-$^2H_3$]acetate as the sole source of carbon. In order to prepare [^{13}C,^2H]griseofulvin, the original mycelium was suspended in medium 3 containing sodium [2-^{13}C,2-$^2H_3$]acetate and cultured for 3 days. Next, to investigate the rate of deuterium

incorporation from medium, the replacement experiment was done in medium 4, containing 50% deuterium oxide and unlabelled sodium acetate. The griseofulvin samples were isolated from the broth after additional incubation of 1 and 12 h, and 1 and 3 days. In addition, another [^{13}C,^{2}H]griseofulvin sample was prepared by the replacement experiment in medium 5, which contained 50% deuterium oxide and sodium [2-^{13}C]-acetate after 3 days incubation.

^{2}H NMR spectra were recorded with a JEOL PFT-100/EC-100 spectrometer operated at 15.28 MHz with proton-decoupled Fourier transform mode. All samples dissolved in chloroform were contained in 10 mm o.d. sample tubes together with a few drops of perfluorobenzene served for ^{19}F frequency control. Spectra were usually taken as spectral width 500 Hz, 2 K data points, 90° pulse (28 μs) and repetition time 2.2 s.

3. Results

3.1. *[^{13}C,^{2}H]griseofulvin from sodium [2-^{13}C,2-$^{2}H_3$]-acetate*

Figure 1A shows ^{2}H NMR spectrum of [^{13}C,^{2}H]griseofulvin from the double-labelled precursor [26–28]. Although the spectral pattern of this sample seems to be quite different from that of [^{2}H]griseofulvin supplemented with [2-$^{3}H_3$]acetate (fig.1C), the spectrum of [^{13}C,^{2}H]griseofulvin can be easily analyzed by taking into account that all of ^{2}H signals of [^{2}H]griseofulvin might split into doublets due to the presence of ^{2}H–^{13}C spin couplings. Thus, we obtained a simulated spectrum (fig.1B) by a procedure of curve-fitting by means of a digital computer, assuming that the individual peaks consist of Lorentzian curves. The peak positions and linewidths were taken from ^{2}H NMR spectrum of [^{2}H]griseofulvin [17] and the ^{2}H–^{13}C spin-coupling constants (by ^{13}C NMR) on the basis of formula: $J_{^{13}C-^{2}H} = (1/6.15)J_{^{13}C-^{1}H}$. It was necessary to include the 3 singlet peaks, marked by the arrows in fig.1A. These singlet peaks are undoubtedly due to ^{2}H–^{12}C, instead of ^{2}H–^{13}C from the double-labelled precursor. The ratios of ^{2}H–^{12}C to ^{2}H–^{13}C are easily calculated from the simulated spectrum (fig.1B): 14.3% (6′-methyl), 30.4% (2′-methoxyl) and 38.5% (4,6-methoxyl). The ratio for 6′-methyl is almost identical to that of the original precursor (12.5% examined from ^{2}H NMR of [2-^{13}C,2-$^{2}H_3$]ace-

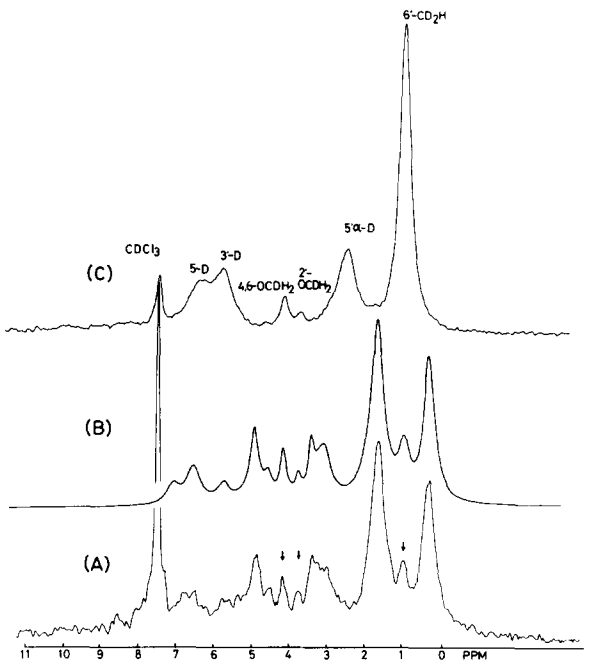

Fig.1. ^{2}H NMR spectra of griseofulvin samples biosynthesized from sodium [2-^{13}C,2-$^{2}H_3$]acetate (A) [medium 3] and from sodium [2-$^{2}H_3$]acetate (C) [medium 2], and computer simulated ^{2}H NMR for A (B). (A) 6.4 mg, 22641 accumulations. (C) 40 mg, 5300 accumulations.
Medium 3: sodium [2-^{13}C,2-$^{2}H_3$]acetate (1 g, 90% ^{13}C, 98% ^{2}H from Prochem); KH_2PO_4 (0.5 g); KCl (50 mg); $MgSO_4 \cdot 7\,H_2O$ (50 mg); and H_2O to make 0.1 l (pH 7.6). Medium 2: sodium [2-$^{2}H_3$]acetate in place of sodium [2-^{13}C,2-$^{2}H_3$]acetate.

Fig.2. ^{2}H NMR spectra of deuterated griseofulvin by medium water (50% D_2O) supplemented with sodium acetate (A–D) [medium 4] and with sodium [2-^{13}C]acetate (E) [medium 5]. (A) After 1 h, 51 mg, d_0 97.9%, 28728 accumulations; (B) after 12 h, 20 mg, d_0 92.2%, 7620 accumulations; (C) after 1 day, 19.1 mg, d_0 80.2%, 6300 accumulations; (D) after 3 days, 13.0 mg, d_0 75.1% 2487 accumulations; (E) 11 mg, 15257 accumulations.
Medium 4: sodium acetate (10 g); KH_2PO_4 (5.0 g); KCl (0.5 g); $MgSO_4\,7H_2O$ (0.5 g); and H_2O : $^{2}H_2O$ (1 : 1) to make 1 l (pH 7.6).
Medium 5: sodium [2-^{13}C]acetate in place of sodium acetate.

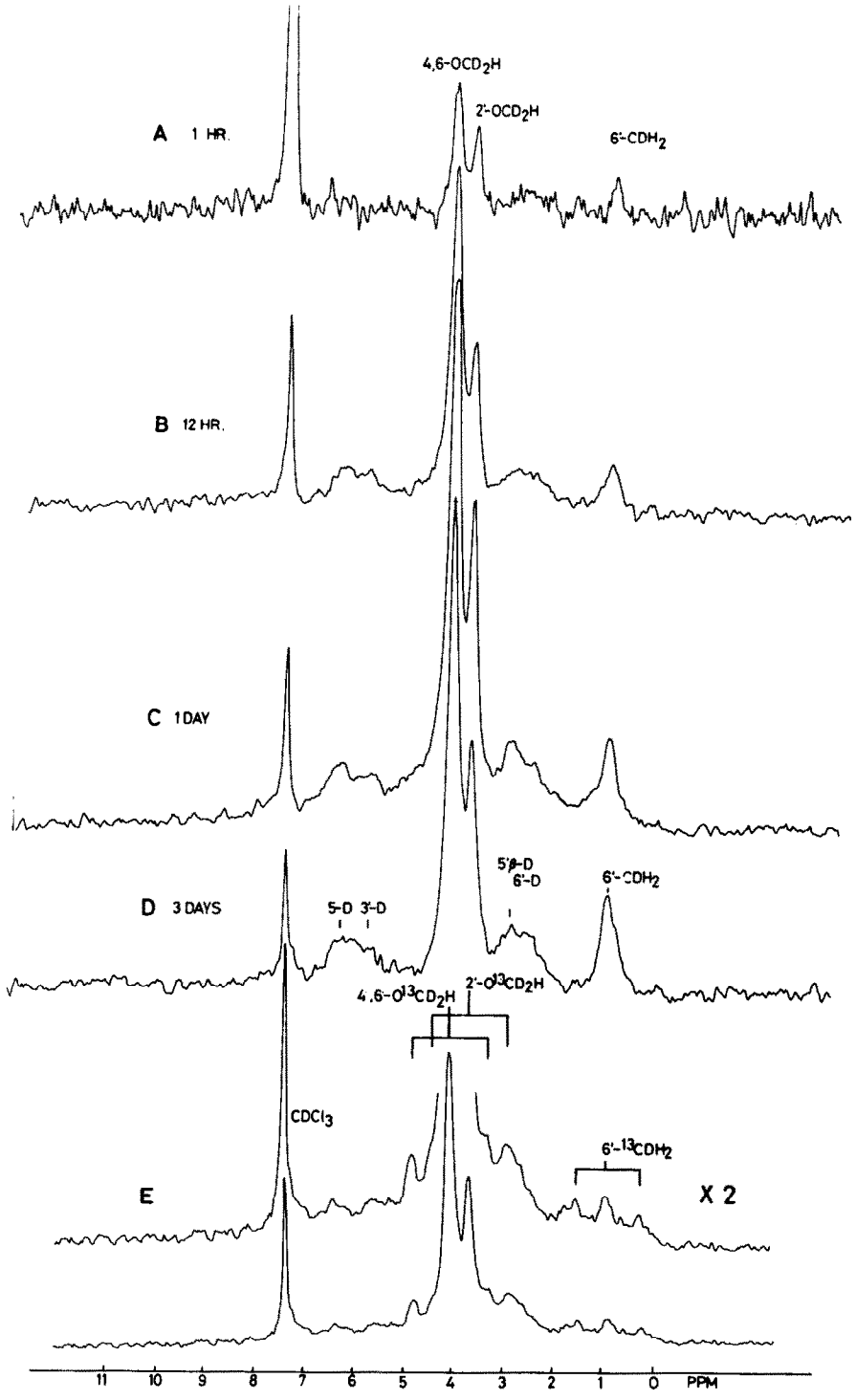
4,6-OCD2H
2'-OCD2H
6'-CDH2
A 1 HR.
B 12 HR.
C 1 DAY
D 3 DAYS
5-D
3'-D
5'β-D
6'-D
6'-CDH2
4,6-O13CD2H
2'-O13CD2H
CDCl3
6'-13CDH2
E
X 2
11 10 9 8 7 6 5 4 3 2 1 0 PPM

Fig.2

tate) but the values for the methoxyl groups are at least 2-times higher than those of the precursor. The latter case may be explained by the fact that the source of the methoxyl group comes from the C-1 pool [14]. Interestingly, it is again found that the peak area of 6′-methyl to 5′-deuterium signals is 2.3 : 1, suggesting that ~1 deuterium atom is replaced by hydrogen from water.

3.2. *[^{2}H]griseofulvin from deuterium oxide and unlabelled sodium acetate*

Figures 2A–D illustrate the time course of incorporation of deuterium from the medium, after the mycelium was transferred to the medium containing 50% deuterium oxide. Interestingly, it is clearly shown that deuterium of 6′-methyl and methoxyl groups comes from the medium even for the sample of very short fermentation time, such as 1 h after being transferred to the medium containing deuterium oxide. This result gives rise to a direct evidence that the 6′-methyl group has the deuterium atoms from the medium. Additionally, ^{2}H NMR signals ascribable to other sites such as 5′β-D, 6′-D, 3′-D and 5-D are also seen in the samples of longer fermentation time (> 12 h). These results are also consistent with the work derived from sodium [2-$^2H_3$]acetate [17].

3.3. *[^{13}C,^{2}H]griseofulvin from deuterium oxide in medium and sodium [2-^{13}C]acetate*

The hydrogen exchange can be visualized more vividly by observation of the ^{2}H–^{13}C spin coupling as a result of covalent-bond formation of deuterium atom(s) from the medium with ^{13}C-labelled nuclei in griseofulvin sample originated from ^{13}C-enriched acetate. Therefore, it is expected that ^{2}H NMR spectrum of this sample might consist of a superposition of ^{2}H NMR spectra of both [^{13}C,^{2}H]- and [^{2}H]griseofulvin samples in view of the above results. In agreement with this expectation, the doublet signals (fig.2E) are thus ascribed to ^{2}H–^{13}C from the ^{13}C-labelled precursor and medium deuterium oxide, and the singlets are due to ^{2}H–^{12}C from unlabelled acetate. The ^{2}H–^{13}C spin-coupling constants thus obtained for 6′-methyl (20 Hz) and methoxyl group (22.4 Hz) are very close to those calculated from the ^{13}C–^{1}H coupling constants (19.3 and 22.5 Hz, respectively).

4. Discussion

The observed ^{2}H NMR peak-intensity of [^{2}H]griseofulvin is 48, 25, 19 and 8%, for 6′-methyl, 5′α-D, 3′-D and 5-D, respectively. The extent of the deuterium incorporation is thus obtained as 1.9 (3), 1 (1), 0.7 (1) and 0.3 (1), respectively, when deuterium for 5′α-D is considered to be fully incorporated and the theoretical values are shown in the parentheses. Thus, we noted that ~1 deuterium atom is replaced for 6′-methyl group by hydrogen in [^{2}H]- and [^{2}H,^{13}C]griseofulvin samples during biosynthesis from the labelled acetate (fig.1). Further, we observed direct incorporation of deuterium from medium water containing 50% deuterium oxide (fig.2). These results strongly support that hydrogen exchange occurs in the 6′-methyl group, which has proven to be the chain-starter group for the heptaketide formation. Naturally, such hydrogen exchange with medium water might be promoted through either intermediate of polyketomethylene (**2**), benzophenones (**3**) or dienones (**4**) forms (scheme). Especially, deuterium at 3′ and 5 position could be replaced by hydrogen in the medium water because of the presence of hydroxyl group(s) at the adjacent *ortho* position(s) in **3**. To some extent, it should also be taken into account that the label of [2-^{13}C]acetate is randomised by the operation of the Krebs cycle, as indicated in [14].

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