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INCORPORATION OF [2-^{3}H]- AND [2-$^{2}H_{3}$]-ACETATE BY PENICILLIUM URTICAE INTO GRISEOFULVIN AND DETERMINATION OF THE STEREOCHEMISTRY OF ISOTOPES AT C-5'

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Griseofulvin is known as a typical example of polyketomethylene antibiotics. The biosynthetic pathway of griseofulvin from acetate-malonate units has already been established by Birch's[1)] and Rhodes's[2)] groups using ^{14}C or ^{36}Cl-precursers. However, problems regarding the extent of incorporation of tritiums or deuteriums from [2-^{3}H]- or [2-$^{2}H_{3}$]-acetate into griseofulvin have remained to be solved.

In this communication we wish to report the incorporation of tritium from [2-^{3}H]-acetate into the griseofulvin molecule by Penicillium urticae. The labelled precursor consisting of 1.08 x 10^{7} dpm of [2-^{14}C]-acetate and 6.77 x 10^{7} dpm of [2-^{3}H]-acetate (^{3}H/^{14}C ratio, 6.27) was added to the shaking culture of P. urticae on the 8th day of the fermentation period. After 2 or 24 hr of fermentation, broth from each flask was filtered to separate the mycelium and filtrate, both of which were subsequently separated into neutral and acidic fractions. Griseofulvin was then obtained from the neutral fraction. The result is shown in Table I.

TABLE I. Radioactivity of Griseofulvin obtained from the Filtrate

Fermentation time (hr)	Isotope	dpm/mg	% of Incorp.	^{3}H/^{14}C
2	^{3}H	7.99 x 10^{3}	0.46	1.85
	^{14}C	4.32 x 10^{3}	1.43	
24	^{3}H	1.29 x 10^{4}	0.55	1.77
	^{14}C	7.27 x 10^{3}	1.78	

If all the possible tritium atoms are incorporated into the griseofulvin molecule without any isotopic exchange, the $^{3}H/^{14}C$ ratio of the griseofulvin should be equal to 6/21 of the $^{3}H/^{14}C$ ratio of the labelled acetate mixture. The result indicated that the biosynthesized griseofulvin (I) did have six tritium atoms on the molecule as shown below

Calcd. $^{3}H/^{14}C$ ratio . 6.27 x 6/21 = 1.79 (for a six tritium atoms incorporation)

Found $^{3}H/^{14}C$ ratio . 1.85 (2 hr fermentation) and 1.77 (24 hr fermentation)

The above results indicate that, during the biosynthesis of griseofulvin through polyketomethylene intermediate (II), all the tritium atoms on griseophenone B (III) were retained in the griseofulvin molecule (Chart 1).

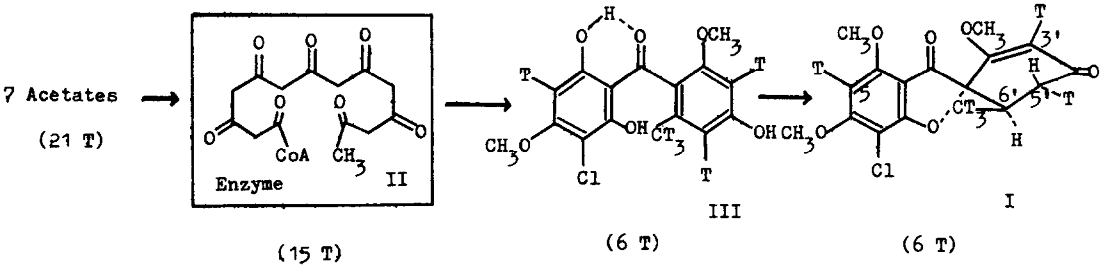

Chart 1. Biosynthesis of Griseofulvin from [2-^{3}H]-Acetate

(Tritium is abbreviated as T in the chart)

The positions of tritium atoms incorporated into griseofulvin were proved by chemical means. Hydrolysis of the doubly labelled griseofulvin ($^{3}H/^{14}C$ ratio, 2.97) with H_2SO_4 in acetic acid$^{3)}$ gave griseofulvic acid (IV), whose $^{3}H/^{14}C$ ratio was reduced to 2.56. This shows that one tritium atom is lost from position 3'. Adsorption of a chloroform solution of griseofulvin ($^{3}H/^{14}C$ ratio, 2.77) on neutral alumina (Woelm, activity I) overnight afforded 5,3'-dichlorogriseofulvin (V), whose radioactivity ratio (1.87) corresponds to the loss of tritiums from positions 5 and 3'. In other experiment, a mixture of griseofulvin and neutral alumina was stirred in chloroform for 16 hr according to the Levine's method. This procedure is known to effect stereoselective partial replacement of 5'β-deuterium of griseofulvin-5',5'-d_2 by hydrogen. The product actually obtained by this experiment, however, showed no change of radioactivity ratio, excluding the possibility of β-orientation of tritium at position 5'. Next, griseofulvin ($^{3}H/^{14}C$ ratio, 2.57) was heated under reflux for 16 hr in $CH_3CH_2CH_2C(CH_3)_2OH$ containing $CH_3CH_2CH_2C(CH_3)_2ONa$, conditions$^{4)}$ known to effect some exchange of both of the C-5' protons. The product (VI, $^{3}H/^{14}C$ ratio, 2.45) indicated a loss of radioactivity corresponding to about 30 % of one tritium atom (Chart 2). This considerably small value (30 %) was proved to be effective for α-orientation of tritium atom at C-5', because the deuteration rates at 5'α of griseofulvin in parallel experiments were about 30 - 50 % of theoretical value on ^1H nmr and mass spectrometric analyses.

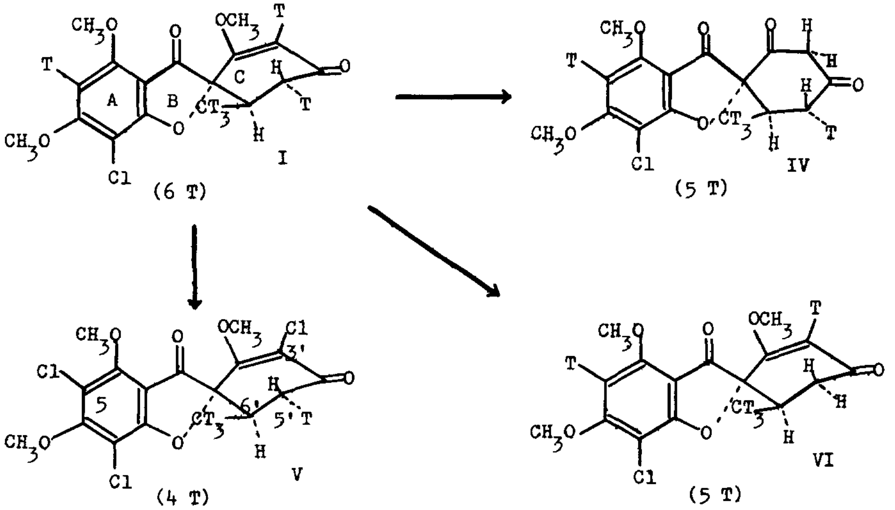

Chart 2. Chemical Transformation of [^3H, ^{14}C]-Griseofulvin

In a separate experiment [2-$^2H_3$]-acetate was fermented with the mycelium of P. urticae in the medium consisting of 5.0 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 15.0 g of sodium [2-$^2H_3$]-acetate·$3H_2O$ (obtained from acetic acid-d_4, 99.5 Atom % D) and H_2O to a total volume of 1 l (pH 7.6). An isotopic composition of [^2H]-griseofulvin thus obtained was proved mass spectrometrically to consist of a deuterated mixture : d_0 61.4, d_1 4.3, d_2 5.9, d_3 8.6, d_4 10.6, d_5 6.4, d_6 1.4, and d_7 and d_8 < 1 %. Using CH_3O signal as a standard, ^1H nmr spectrum was examined for the decrease in each ^1H signal intensity. The positions of deuteriums were 5, 3', 5'α and 6'-CH_3, and the incorporation ratio at each position was about 17 %.

Based on the present data, it was concluded that the positions of tritium or deuterium atoms incorporated into griseofulvin are 5 in the A ring, and 3', 5'α, and 6'-methyl in the C ring. This result indicates the trans diaxial type reduction on C ring during the course of griseofulvin biosynthesis.

This investigation proves that the polyketomethylene intermediate, if present, is protected from isotopic exchange in aqueous medium before being forewarded to the next transformation on a multienzyme complex.

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