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THE BIOSYNTHESIS OF PATULIN: THE MECHANISM OF OXIDATIVE AROMATIC RING CLEAVAGE AND LOSS OF SIDE CHAIN PROTONS FROM AROMATIC INTERMEDIATES

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Method to detect the incorporation of ^{18}O and ^{2}H by isotope shift in ^{13}C-NMR spectroscopy was applied to study the mechanism of patulin biosynthesis in *Penicillium patulum* NRRL 2159A. From the results of incorporation experiments with $^{18}O_2$ and [1-^{13}C, $^{18}O_2$]-acetate, monooxygenase appeared to be involved in the ring cleavage of an aromatic intermediate. ^{2}H from [2-^{13}C, 2-$^{2}H_3$]-acetate was detected at C-5, but not at C-1, indicating that the side chain protons of the aromatic intermediate were lost in the course of patulin biosynthesis.

KEYWORDS —— patulin; *Penicillium patulum*; ^{13}C-NMR; isotope shift; $^{18}O_2$; [1-^{13}C, $^{18}O_2$]-acetate; [2-^{13}C, 2-$^{2}H_3$]-acetate; monooxygenase

Patulin is one of the most commonly occurring mycotoxins produced by a number of *Penicillia* and *Aspergilli* and its biosynthesis has been studied by many workers.[1] Tanenbaum *et al.* proved by a feeding experiment with [^{14}C]-6-methylsalcylic acid (6MS) that 6MS was incorporated into patulin and the oxidative ring cleavage of an aromatic intermediate was involved in patulin biosynthesis.[2] The biosynthetic pathway revealed by tracer experiments[3] is shown in Fig.1. The several enzymes responsible for patulin biosynthesis have been extensively studied[4] and gentisaldehyde is so far

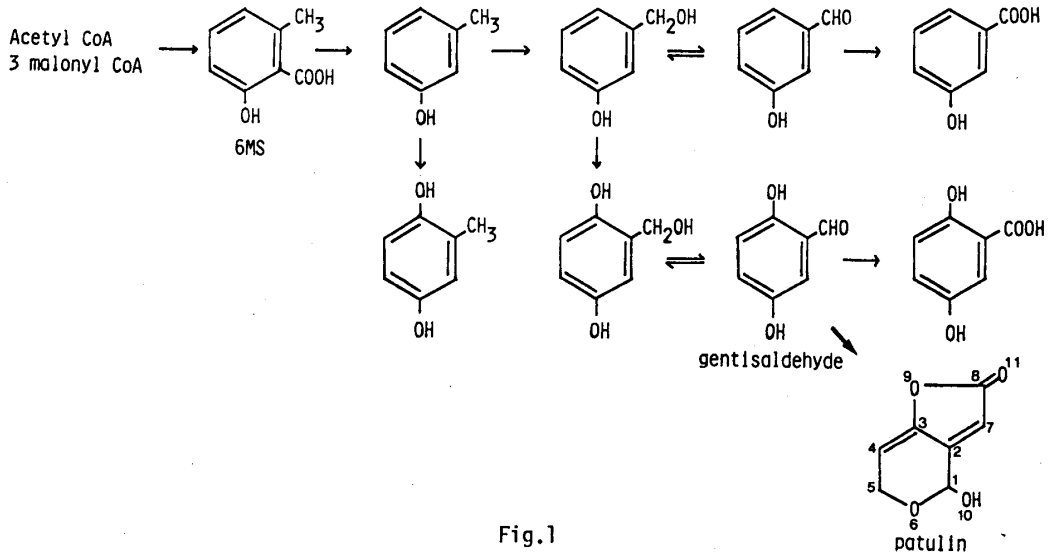

Fig.1

regarded as a final aromatic intermediate leading to patulin.

However the mechanism of the ring cleavage of the aromatic intermediate has not been fully clarified. Scott *et al.* suggested a dioxygenase mechanism for the ring cleavage of gentisaldehyde,[5] while Gaucher *et al.* preferred a monooxygenase mechanism (Fig.2).[6] We carried out feeding experiments with $^{18}O_2$ gas and [1-^{13}C, $^{18}O_2$]-acetate to clarify which mechanism is actually involved in patulin biosynthesis.[7]

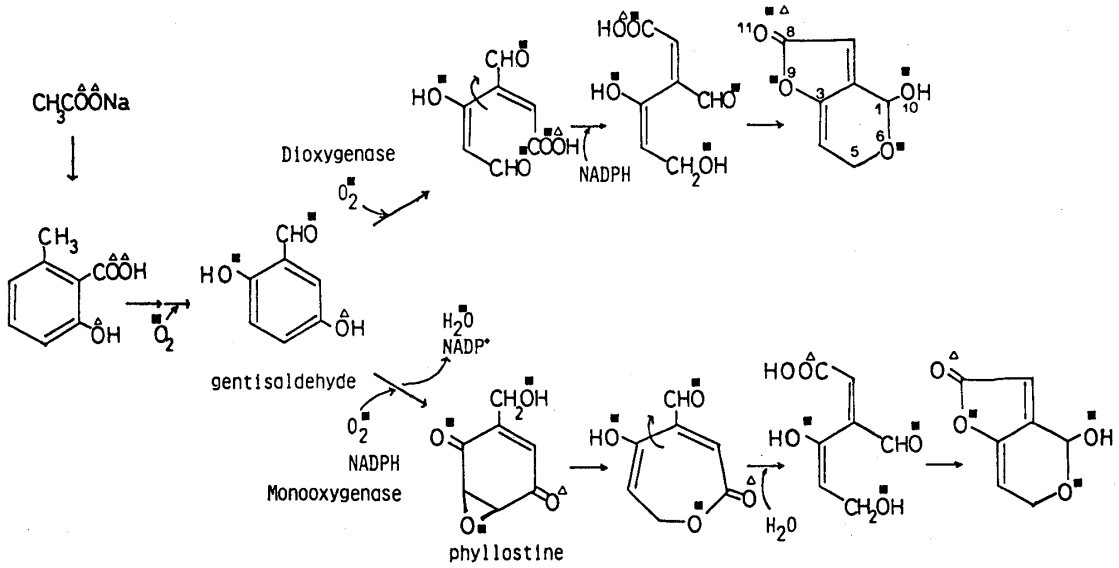

Fig.2

A shake culture of *P. patulum* NRRL 2159A was grown in 150 ml of Czapek-Dox medium in a 500 ml flask. Twenty mg each of sodium [1-^{13}C]-acetate and sodium [2-^{13}C]-acetate was administered to 27 h old culture. At the same time, $^{18}O_2$ gas, diluted to 20% (v/v) with nitrogen gas, was introduced (80 ml/h). The culture was harvested at 53 h and patulin was purified by silicagel chromatography followed by recrystallization from chloroform. Satisfactory incorporation ratio of acetate (8 to 10% per labelled site) was observed in every experiment.

The ^{13}C-NMR spectra were measured at 100.7 MHz with 16 K data points for an observation range of 2 KHz for each carbon and zerofilled to 32 K. The incorporation of ^{18}O was detected by ^{13}C-NMR spectra through the upfield isotope shift of ^{13}C directly attached to ^{18}O.[8] ^{18}O was incorporated into O-6, O-9 and O-10 since C-1, C-3, C-5 and C-8 showed ^{13}C-^{18}O signals as they appear in Fig.3. The isotope shift values for C-1, C-3, C-5 and C-8 were 0.072, 0.012, 0.016 and 0.012 ppm, respectively. The shifted signal of C-1 was broad and its shift value (0.072 ppm) was unusually larger than those of the other carbons bearing one ^{18}O atom. The results indicate that two ^{18}O atoms are attached to C-1. The isotope shift of C-8 was considered to be induced by the presence of ^{18}O at O-9 because the isotope shift due to O-11 was 0.035 ppm as discussed below. The coincidence of the shift values of C-3 and C-8 (0.012 ppm) also supported the above findings.

The isotope shift of C-8 caused by ^{18}O-11 was observed in the ^{13}C-NMR of patulin labelled with [1-^{13}C, $^{18}O_2$]-acetate[9] and the retention of ^{18}O was not observed at O-6, O-9 and O-10 as expected. Spectrum A in Fig.4 is a proton noise decoupled signal of C-8 at 100.7 MHz in which long range ^{13}C-^{13}C couplings were observed as the results of multiple incorporation of labelled acetate in a molecule. These long range ^{13}C-^{13}C couplings were eliminated by spin echo technique employing pulse sequence of (90°-τ-180°-τ-acquisition-pulse delay) where τ was set to 1/4J (Fig.4, B).[10]

The result of this pair of experiments indicates that O-11 was derived not from molecular oxygen but from acetate oxygen and supports the monooxygenase mechanism shown in Fig. 2. The

retention of the intact C-O bond from acetate was *ca.*15%, indicating that considerable oxygen exchange took place in the course of patulin biosynthesis. Hutchinson *et al.* observed such oxygen exchange in their studies on lasalocid A biosynthesis and postulated that oxygen exchange took place during polyketide cyclization, most likely at the stage of enzyme bound thioesters.[11] However another possibility, that oxygen exchange occurred at the stage of a hypothetical intermediate formed by the ring cleavage of the aromatic intermediate, can not be excluded.

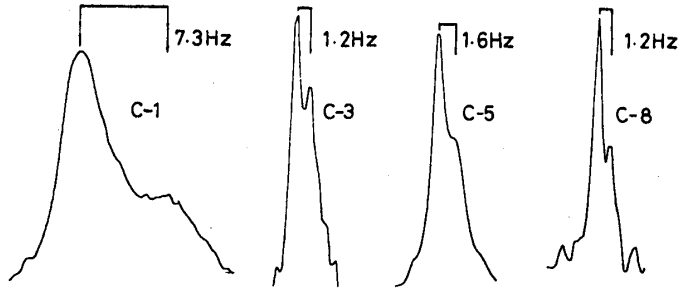

Fig.3

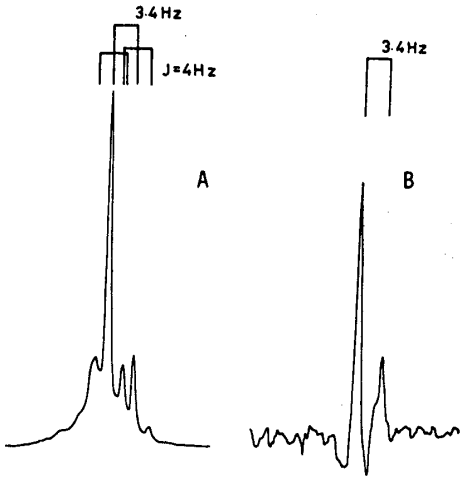

Fig.4

Scott *et al.* reported that the side chain protons of aromatic precursors such as *m*-hydroxybenzyl alcohol, gentisyl alcohol and gentisaldehyde were not incorporated into patulin.[3c] On the other hand Staunton *et al.* recently observed that 95% of ^{2}H was retained in the methyl group of 6MS derived from [1-^{13}C, 2-$^{2}H_3$]-acetate in *Penicillium griseofulvum*.[12] Feeding experiment using [2-^{13}C, 2-$^{2}H_3$]-acetate was therefore carried out to investigate the fate of the side chain protons of aromatic intermediates, since they are converted from methyl protons of 6MS (Fig.5). The ^{2}H noise decoupled 100.7 MHz ^{13}C-NMR of patulin labelled with [2-^{13}C, 2-$^{2}H_3$]-acetate indicated

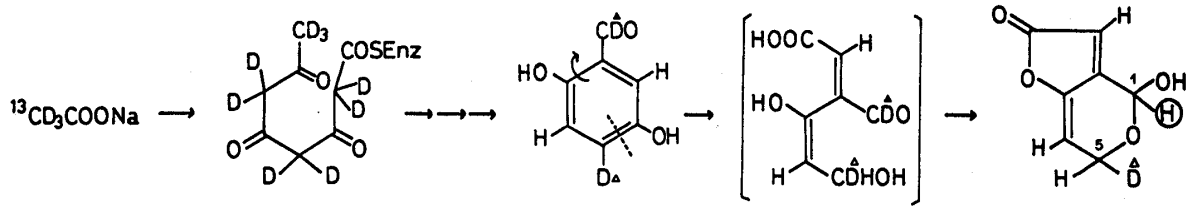

Fig.5

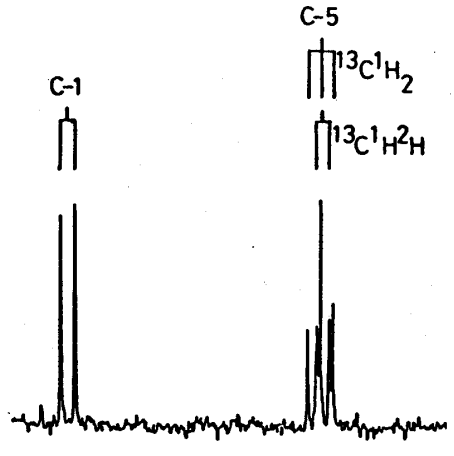

Fig.6

Table. ^{13}C-NMR Chemical Shifts of Patulin
(ppm, TMS / d_6-acetone)

C-1	C-2	C-3	C-4	C-5	C-7	C-8
89.7	153.0	147.3	109.2	60.1	110.9	169.5
d	s	s	d	t	d	s

s:singlet, d:doublet, t:triplet
(multiplicity in off-resonance spectra)

retention of ^{2}H at C-5, while C-1 did not show the ^{13}C-^{2}H signal (Fig.6). The side chain protons are originally derived from the starter acetate unit, while the protons of the aromatic ring from the malonyl units. They are considered to be more easily exchangeable with environmental protons than those of the starter acetate unit. Thus the retention of ^{2}H at C-5 strongly indicated that the loss of the side chain protons occurred at the stage of the aromatic intermediates. C-5 showed a doublet signal of $^{13}C^{1}H^{2}H$ at 0.408 ppm upfield to a triplet signal of $^{13}C^{1}H_{2}$, indicating that one of the two protons was derived from acetate hydrogen. Since the intermediate role of gentisic acid in patulin biosynthesis has been excluded from the results of the feeding experiment employing carboxyl-^{14}C labelled specimen,[2] the mechanism for the loss of ^{2}H remains unclear.

The ^{13}C-NMR assignments of patulin are summarized in Table. The assignments were based on ^{13}C enrichment in patulin labelled with [1-^{13}C, $^{18}O_{2}$]-acetate and [2-^{13}C, 2-$^{2}H_{3}$]-acetate. The assignment previously reported [6a] should be corrected.

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