BIOSYNTHESIS OF CITIRININ IN *ASPERGILLUS TERREUS* INCORPORATION STUDIES WITH [2-^{13}C,2-^{2}H$_3$], [1-^{13}C,^{18}O$_2$] AND [1-^{13}C,^{17}O] ACETATE

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Asbstract: Incorporation of ^{2}H and ^{18}O of [2-^{13}C,2-^{2}H$_3$] and [1-^{13}C,^{18}O$_2$] acetate into citrinin(1) was confirmed by using ^{13}C-NMR, and incorporation of ^{17}O from [1-^{13}C,^{17}O] acetate was also detected by ^{17}O-NMR.

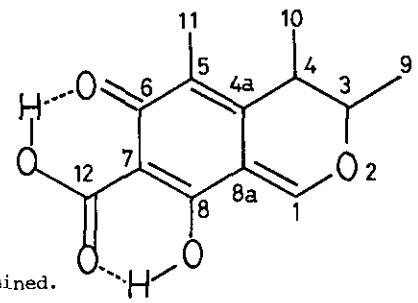
Citrinin(1), a pentaketide mycotoxin produced by fungi belonging to *Aspergilli* and *Penicillia*,[1] is known to be biosynthesized from one acetyl CoA, four malonyl CoA and three C_1 units.[2] Studies on the intermediary stage of the biosynthesis with advanced precursors suggested a keto-aldehyde(2) as an immediate intermediate released from the enzyme template of polyketide biosynthesis.[3] In the present studies multiple labelled precursors, [2-^{13}C,2-^{2}H$_3$],[4] [1-^{13}C,^{18}O$_2$] and [1-^{13}C,^{17}O] acetate, were administered to the culture of *Aspergillus terreus* Thom. ATCC 24839 to show the possible utilization of these precursors for tracing the fate of acetate hydrogen and oxygen.

Staunton *et al.* discussed the ^{13}C-NMR spectrum of citrinin(1) on the basis of the ^{13}C-NMR spectrum of citrinin(1) labelled with [1,2-^{13}C$_2$] acetate.[5] In view of possible obscurity involved in the assignment of C-5,6,7 and 8 given by Staunton *et al.*, the authors performed single frequency decoupling to C-11 methyl protons. When the C-11 methyl protons(δ 2.02) were irradiated selectively, 1.7 to 3.6-fold enhancements in intensity was observed in the ^{13}C-NMR signals at 122.6, 139.2 and 183.7 ppm, whereas no considerable changes in the signals at 100.0, 107.1 or 177.2 ppm. Since enhancement in signals in single frequency decoupling is caused by the nuclear Overhauser effect(NOE) and also by the disappearance of long-range couplings, the previous assignment for the two pairs of signals(C-5,6 and C-7,8),[5] which are derived from the same acetate units, should be alternated as shown in Table 1.

Carbon	Chemical shift (ppm)	$^{1}J_{C-C}$(Hz)*	Signal enhancement on irradiation at δ 2.02(fold)
1	162.9	69.6	-†
3	81.8	37.8	-
4	34.5	40.9	-
4a	139.2	40.9	1.7
5	122.6	56.9	3.6
6	183.7	56.9	2.5
7	100.0	63.6	1.0
8	177.2	63.6	1.0
8a	107.1	69.6	1.0
9	18.2	37.8	-

* Coupling constants reported by Staunton *et al.* †Not determined.

Table 1. Assignment for ^{13}C-NMR of citrinin(1).



Citrinin (1)

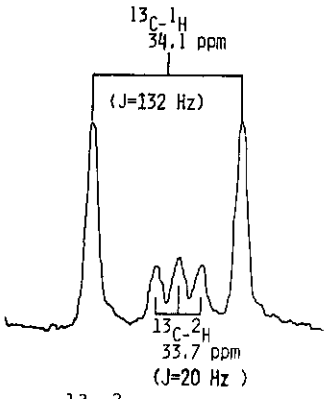


Fig.1 ^{13}C-^{2}H signal of C-4 of citrinin(1) labelled with [2-^{13}C,2-$^{2}H_3$] acetate.

Carbon	Chemical Shift (ppm)	^{13}C-^{18}O isotope shift(Hz) 25.05MHz	50.31MHz	100.7MHz
1	162.9	- *	- *	- *
3	81.8	1.1	2.1	4.2
6	183.7	1.1	1.5	3.9
8	177.2	1.1 ±(0.38)†	1.7 ±(0.60)†	3.9 ±(0.25)†

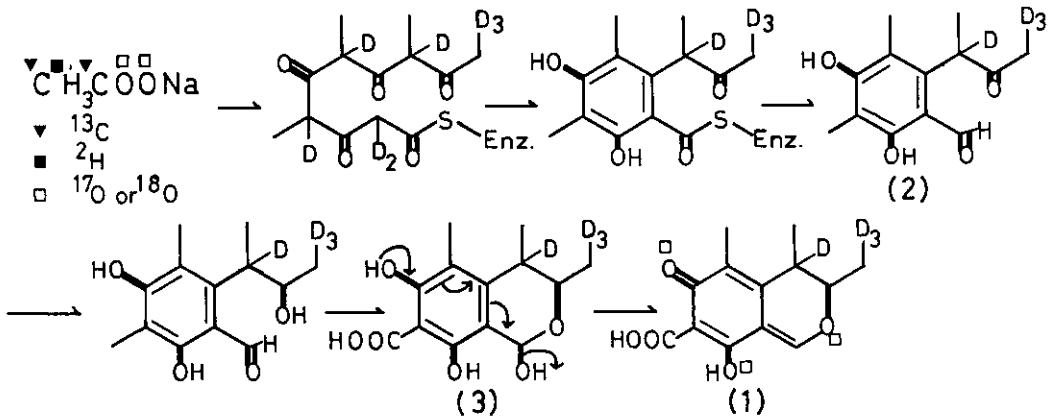
* ^{13}C-^{18}O signals were not observed.
† Errors were calculated from data points.

Table 2. ^{13}C-^{18}O signals observed in ^{13}C-NMR spectra of citrinin (1) labelled with [1-^{13}C,$^{18}O_2$] acetate.[11]

The precursors were separately added daily to the stationary culture of *A. terreus* on a modified Czapek-Dox medium for 10 days starting on 11th day after inoculation. Three to six days after the final administration, the cultures were harvested and citrinin(1) was isolated by EtOAc extraction and column chromatography on acidified silica gel.[6] The ^{1}H-coupled ^{13}C-NMR spectrum of citrinin(1) labelled with [2-^{13}C,2-$^{2}H_3$] acetate showed a triplet signal(^{13}C-^{2}H; J=20 Hz) centered at 33.7 ppm between the doublet of ^{13}C-^{1}H signal of C-4 as shown in Fig. 1. This fact undoubtedly demonstrates the incorporation of ^{2}H into C-4,[5] as suggested by Staunton *et al.*[5] An upfield shift by 0.4 ppm is well in accord with the value of isotope shift induced by substitution with one ^{2}H.[4] The incorporation of ^{2}H into the starting methyl group of polyketide(C-9) was also evidenced by a marked decrease in signal intensity in comparison with that of citrinin(1) labelled with [2-^{13}C] acetate.[7]

^{18}O isotope shift in ^{13}C-NMR was first reported by Risley and Van Etten in [^{18}O]*t*-butanol[8] and the values of ^{18}O isotope shift of 32 labelled compounds by Vederas.[9] Thus, the authors investigated the incorporation of acetate oxygen into citrinin(1) by using [1-^{13}C,$^{18}O_2$] acetate as a precursor to trace the fate of acetate oxygen in polyketide biosynthesis. [1-^{13}C, $^{18}O_2$] acetate was prepared by an exchanging method from [1-^{13}C] acetate(90 atom%) and [^{18}O] water(99 atom %),[10] and the completion of the exchange reaction was confirmed by the presence of a single peak at 178.57 ppm in ^{13}C-NMR(25.05 MHz). A mixture of [1-^{13}C,$^{18}O_2$] and [1-^{13}C,$^{16}O_2$] acetate showed two peaks at 178.57 and 178.63 ppm. ^{18}O isotope shift of acetate is 1.5 Hz. The ^{1}H-decoupled ^{13}C-NMR spectrum(50.31 MHz) of citrinin(1) labelled with [1-^{13}C,$^{18}O_2$] acetate showed ^{13}C-^{18}O signals for C-3, 6 and 8, but not for C-1 as shown in Table 2.[11] It is noteworthy that the ^{13}C-^{18}O signals were also detected in the ^{13}C-NMR spectrum(Fig.2a) recorded on a 25.05 MHz spectrometer with 32 K data points and 6000 Hz spectral width. In order to obtain accurate shift values the ^{13}C-NMR spectrum of ^{18}O-labelled citrinin(1) was measured with a 100.7 MHz spectrometer with 32 K data points and 4000 Hz spectral width. The ^{13}C-^{18}O signals of C-3, 6 and 8 showed upfield shift by 4.2, 3.9 and 3.9 Hz, respectively, as shown in Fig.2b and Talbe 2.[11] The foregoing results clearly demonstrate the integrety of ^{13}C-^{18}O bonds at C-3, 6 and 8, whereas ^{13}C-^{18}O bond at C-1 was cleaved in the course of biosynthesis, indicating that the quinone methide structure of citrinin (1) is formed by the elimination of hemi-acetal hydroxyl as shown in the structure(3). Recently, Vederas *et al.* reported the incorporation of [$^{18}O_2$] oxygen and [1-^{13}C,$^{18}O_2$] acetate into averufin and cytochalasin.[12]

An incorporation experiment with [1-^{13}C,^{17}O] acetate, which was prepared from [1-^{13}C] acetate (90 atom%) and [^{17}O] water(30 atom%), also gave positive results. The ^{17}O signal of the starting labelled acetate was observed at 282 ppm in the ^{17}O-NMR spectrum(12.15 MHz; natural abundance



Scheme of biosynthesis of citrinin(1) from [2-^{13}C,2-^{2}H$_3$],[1-^{13}C,^{18}O$_2$] and [1-^{13}C,^{17}O] actate

$H_2{}^{17}O$ as a standard) and the incorporation of ^{13}C into citrinin(1) was confirmed by ^{13}C-NMR(25.05 MHz). In the ^{17}O-NMR spectrum(54.26 MHz), three ^{17}O signals of the labelled citrinin(1) were observed at 148, 179 and 279 ppm as shown in Fig. 3 and tentatively assigned according to published data.[13] ^{17}O chemical shift values are very sensitive to the changes of electron density on oxygen atoms. The chemical shift value of C-6 oxygen(279 ppm) is smaller than those of normal carbonyl groups, and those of C-8 oxygen and O-2(179 and 148 ppm) are larger than those of phenol and enol groups.[13] These facts may be explained by keto-enol tautomerism of citrinin(1a and 1b) and also by the quinone methide(extended quinone) structure of citrinin(1). At present, the data of ^{17}O

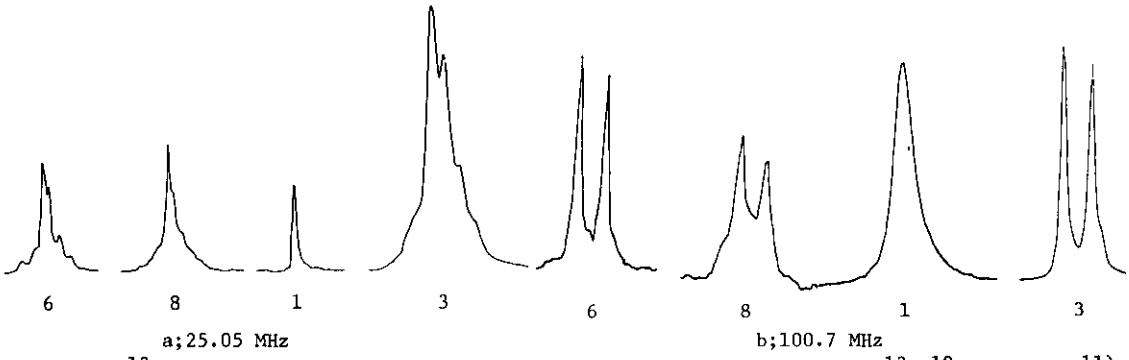


Fig.2 ^{13}C-NMR spectra of C-1,3,6 and 8 of citrinin(1) labelled with [1-^{13}C,^{18}O$_2$] acetate.[11]

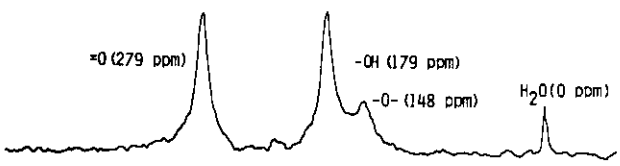
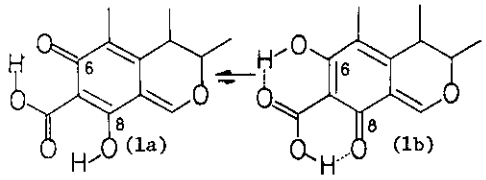


Fig.3 ^{17}O-NMR power spectrum of citrinin(1) labelled with [1-^{13}C,^{17}O] acetate.[14]



Keto-enol tautomerism of citrinin(1).

chemical shift are limited to simple compounds and we are currently studying relationship between ^{17}O chemical shifts and the results of X-ray analysis. This is the first case that ^{17}O-NMR was successfully applied in biosynthetic studies, and further works on the utilization of [^{13}C,^{2}H], [^{13}C,^{18}O], [^{13}C,^{17}O] and [^{13}C,^{15}N] labelled precursors in biosynthesis are in progress in our laboratories.

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11) The ^{13}C-NMR spectra were recorded on a JEOL FX-100, a Varian XL-200 or a JEOL FX-400 NMR spectrometer. The ^{17}O-NMR spectra were recorded by using a JEOL FX-90 or a JEOL FX-400 NMR spectrometer.

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