

BIOSYNTHESIS OF CITRININ IN *ASPERGILLUS TERREUS*

INCORPORATION STUDIES WITH [2-¹³C, 2-²H₃], [1-¹³C, ¹⁸O₂] AND [1-¹³C, ¹⁷O]-ACETATE¹

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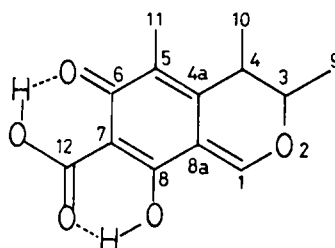
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Abstract—Incorporation of ²H and ¹⁸O of [2-¹³C, 2-²H₃] and [1-¹³C, ¹⁸O₂]-acetate into citrinin (1) was detected with ¹³C-NMR through ²H-¹³C coupling and isotope shift induced by ²H and ¹⁸O. Another possible multiple labelled acetate, [1-¹³C, ¹⁷O]-acetate, was tested for its potentiality as a precursor to trace the fate of acetate oxygen. ¹³C-¹⁷O Coupling in ¹³C-NMR was too small to be detected, however the incorporation of ¹⁷O was directly measured with ¹⁷O-NMR. Unusual ¹⁷O chemical shift values of citrinin (1) were accounted for by the presence of keto-enol resonance forms which was verified by the C-O bond lengths determined by X-ray analysis.

Citrinin (1), a yellow crystalline compound first isolated from *Penicillium citrinum*, is produced by a large number of fungi belonging to *Penicillium* and *Aspergillus*.² It showed broad antibiotic activity, but its nephrotoxic properties prevented its application as a drug.³ Extensive feeding and degradation studies have shown that citrinin (1) is derived from one acetyl CoA, four malonyl CoA and three C₁ units.⁴⁻⁶ An assembly pattern of five acetate units was unambiguously shown by an incorporation experiment with [1, 2-¹³C₂]-acetate.⁷ An investigation of metabolites of *Penicillium citrinum* and of its mutants was carried out to find intermediates of citrinin (1) biosynthesis, however all the isolated compounds were not the intermediates of biosynthesis.⁸ Recently, the intermediary stage of citrinin (1) biosynthesis has been investigated with labelled advanced precursors and the results obtained suggest that a keto-aldehyde (2), 4,6-dihydroxy-3,5-dimethyl-2-(1-methyl-2-oxopropyl)-benzaldehyde, is an immediate intermediate released from the enzyme template of polyketide biosynthesis.⁹⁻¹³ In previous papers, we reported the application of multiple labelled acetate, [2-¹³C, 2-²H₃], [1-¹³C, ¹⁸O₂] and [1-¹³C, ¹⁷O]-acetate, as tracers in the biosynthetic studies of polyketides.¹⁴⁻¹⁹ This paper describes the full details of studies on citrinin (1) biosynthesis with multiple labelled acetate. Discussion will be made on the scope and limitation of ¹⁸O and ¹⁷O labelled acetate in biosynthetic studies.

Staunton *et al.* briefly discussed the ¹³C-NMR spectrum based on the ¹³C-¹³C coupling data of citrinin (1) enriched with [1, 2-¹³C₂]-acetate.⁷ In view of possible obscurity involved in the assignment of C-5, C-6, C-7 and C-8 given by Staunton *et al.*, the authors performed single frequency decoupling to C-11 Me protons. When the C-11 Me protons (δ 2.02) were irradiated selectively, the intensities of signals at

122.6, 139.2 and 183.7 ppm increased 3.6, 1.7 and 2.5-fold, respectively, while those at 100.0, 107.1 and 177.2 ppm remained unchanged. Enhancement of signal intensity in selective decoupling is caused by the disappearance of long range coupling and by the nuclear Overhauser effect (NOE). The two pairs of carbons (C-5, 6 and C-7, 8) deriving from the same acetate units should be alternated from the assignment given by Staunton *et al.*⁷ The signals at 122.6 and 183.7 ppm showing 3.6 and 2.5-fold enhancement in signal intensities upon selective irradiation at C-11 protons should be assigned to C-5 and C-6, respectively, because they should be closer to C-11 than those at 100.0 and 177.2 ppm.



Citrinin (1)

Based on this assignment we tested the potentiality of multiple labelled acetate in obtaining further information on the biosynthesis of citrinin (1) through the detection of ²H, ¹⁷O and ¹⁸O incorporation. Various kinds of multiple labelled acetate were mixed with [1-¹⁴C]-acetate and separately added daily for ten days to stationary cultures of *Aspergillus terreus* (ATCC 24839) grown on a modified Czapek-Dox

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

Carbon	Chemical shift	$^1J_{\text{C-C}}(\text{Hz})^a$	Signal enhancement	
	(ppm)		on irradiation at	2.02 (fold)
1	162.9	69.6	-	^b
3	81.8	37.6	-	-
4	34.5	40.9	-	-
4a	139.2	40.9	-	-
5	122.6	56.9	1.7	-
6	183.7	56.9	3.6	-
7	100.0	63.6	2.5	-
8	177.2	63.6	1.0	-
8a	107.1	69.6	1.0	-
9	18.2	37.8	-	-
10	9.4	-	-	-
11	18.4	-	-	-
12	174.1	-	-	-

^a Coupling constants reported by Staunton *et al.*⁷

^b Not determined.

medium. The cultures were harvested 2–6 days after the final administration of the labelled compounds. Labelled citrinin (1) was isolated from acidified culture medium by ethyl acetate extraction followed by a column chromatography on acidified silica gel. One culture flask containing 150 ml medium yielded approx. 150 mg crude citrinin (1) and final yields after recrystallization were 30–50 mg (Table 2).

The incorporation of ^2H from $[2-^{13}\text{C}, 2-^2\text{H}_3]$ -acetate into C-4 and C-9 hydrogen was readily demonstrated by a marked decrease of signal intensity in the ^{13}C -NMR spectrum of citrinin (1) labelled with $[2-^{13}\text{C}, 2-^2\text{H}_3]$ -acetate in comparison with those of citrinin (1) labelled with $[2-^{13}\text{C}]$ -acetate. From the decrease of signal intensities the ratios of ^2H retention vs ^{13}C at C-4 and C-9 were calculated to be 41 and 89%, respectively. A higher ^2H retention at C-9 is a result of the reaction mechanism of polyketide biosynthesis. C-9 is derived directly from acetyl CoA forming a starter unit, while C-4 is introduced *via* malonyl CoA and present in a middle of polyketide chain, indicating much higher possibility of exchange of hydrogen. In the ^1H -non-decoupled ^{13}C -NMR spectrum (25.05 MHz) ^{13}C - ^2H signal of C-4 was directly observed as a triplet ($J = 20$ Hz) centered at 33.7 ppm between a ^{13}C - ^1H doublet ($J = 132$ Hz) centered at 34.1 ppm (Fig. 2). An upfield shift by 0.4 ppm is a normal isotopic shift value of ^{13}C bearing one ^2H . However, the field covering the ^{13}C - ^2H signals

of C-9 showed too complicated signals to be interpreted. The results are well in accord with those suggested by Staunton *et al.*, who observed the incorporation of acetate hydrogen by using ^2H -water for culture medium.⁷

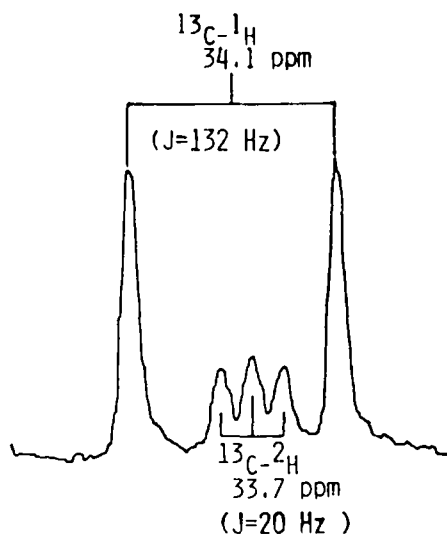


Fig. 1. ^2H - ^{13}C Signal of C-4 in the ^1H -non-decoupled ^{13}C -NMR spectrum (25.05 MHz) of citrinin (1) labelled with $[2-^{13}\text{C}, 2-^2\text{H}_3]$ -acetate.

Table 2. Feeding experiments with multiple labelled acetate and incorporation into citrinin (1)

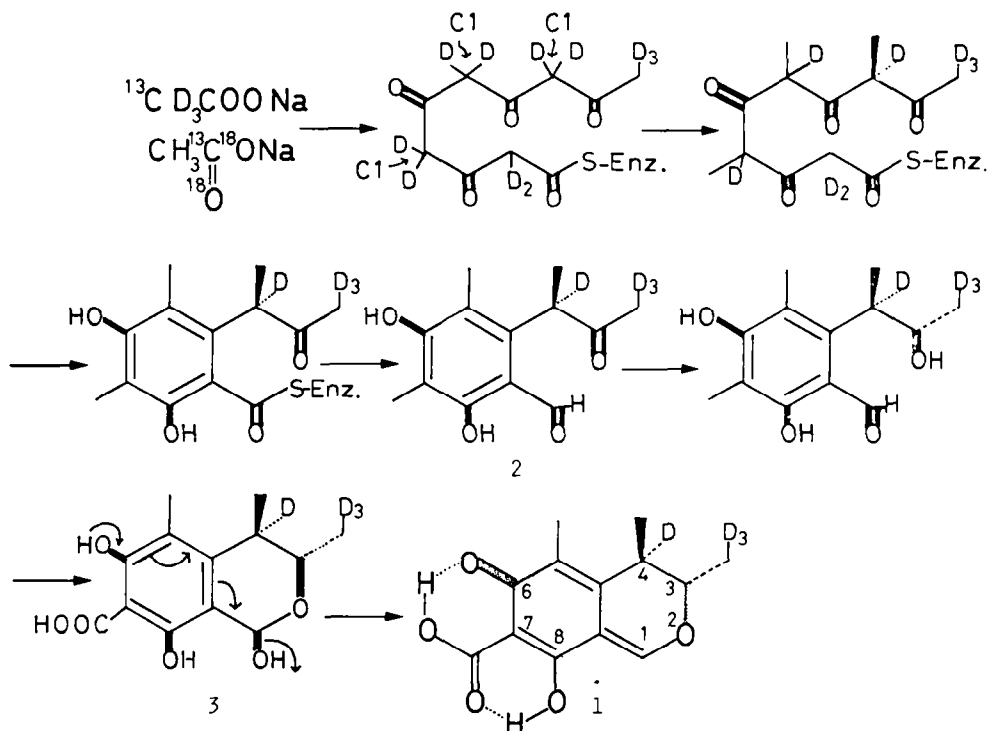
No acetate	Feeding days	Harvested day	Yield(mg)	Enrichment %/C
1 $[1-^{13}\text{C}]$ 100 mg	9th–19th	21st	46	7.3
2 $[2-^{13}\text{C}]$ 100 mg	10th–21st	27th	33	4.8
3 $[2-^{13}\text{C}, 2-^2\text{H}_3]$ 100 mg	10th–21st	27th	32	5.2
4 $[1-^{13}\text{C}, ^{18}\text{O}_2]$ 150 mg	10th–19th	22nd	51	9.8
5 $[1-^{13}\text{C}, ^{18}\text{O}_2]$ 25 mg non-labelled acetate 75 mg	10th–19th	21st	50	1.5 ^a
6 $[1-^{13}\text{C}, ^{17}\text{O}]$ 100 mg	11th–19th	26th	46	6.7

^a Calculated from specific incorporation ratio of ^{14}C based on the amount of ^{13}C .

Next, we studied the origin of O atoms of citrinin (1). It contains three O atoms possibly derived from acetate. Risely and Van Etten demonstrated that ^{18}O induced isotope shift in ^{13}C -NMR could apply in the study of O exchange kinetics in an acid catalyzed exchange of *t*-butanol OH group.²⁰ This was followed by the reports of Vederas *et al.* and Risely and Van Etten. They reported the values of ^{18}O induced isotope shift of ^{13}C signals of various compounds.²¹⁻²⁶ Their earlier reports tempted us to explore the possibility to use $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate as a precursor to detect the integrity of ^{13}C - ^{18}O bond of acetate in polyketide biosyntheses. Since the incorporation ratios of labelled acetate into citrinin (1) were particularly high, an incorporation experiment with $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate was carried out as a case study for tracing the fate of acetate oxygen. $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate was prepared by an exchanging method from $[1-^{13}\text{C}]$ -acetate (90 at. %) and $[^{18}\text{O}]$ -water (99 at. %).^{27,28} In order to determine the isotopic composition of this labelled acetate, $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate was converted into a *p*-phenylphenacyl ester and measured a high resolution mass spectrum. This labelled acetate consisted of 74.6% of $[1-^{13}\text{C}, ^{18}\text{O}_2]$ and 16.4% of $[1-^{13}\text{C}, ^{18}\text{O}]$ -acetate.

Thus prepared $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate was mixed with unlabelled acetate and measured ^{13}C -NMR spectrum (25.05 MHz) to clarify if it is possible to detect shifted signal induced by ^{18}O with a 25.05 MHz spectrometer. The ^{13}C - ^{18}O signal of the labelled acetate was observed 1.5 Hz upfield to the corresponding ^{13}C - ^{16}O signal. This result encouraged us to attempt detecting ^{18}O incorporation with a 25.05 MHz ^{13}C -NMR spectrometer. $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate was pulsedly added to the culture of *A. terreus* as in the case of $[2-^{13}\text{C}, 2-^2\text{H}_1]$ -acetate. We feel the figures of

enrichment per carbon, calculated from the specific incorporation ratios of ^{14}C without taking any consideration for randomization, are always higher than those observed in ^{13}C -NMR spectra. Although shifted signals were accompanied by several small satellites, the ^{13}C - ^{18}O signals were clearly detected at C-3, C-6 and C-8 in the ^{13}C -NMR spectrum (25.05 MHz) recorded under a conventional measuring condition of 6000 Hz spectral width and 32 K data points (Fig. 2a). The same sample of enriched citrinin (1) was then submitted for a measurement with a 50.31 MHz NMR spectrometer with an expectation to observe better resolved shift signals. The spectrum was recorded under a conventional measuring condition of 10,000 Hz spectral width and 32 K data points. The signals corresponding to C-1, C-3, C-6 and C-8 are shown in Fig. 2(b). The results clearly demonstrate that the integrity of ^{13}C - ^{18}O bonds at C-3, C-6 and C-8 was maintained and that the quinone methide structure of citrinin (1) is formed by the elimination of a hemiacetal OH at C-1 in an intermediate (3). A biosynthetic scheme proposed by Staunton *et al.* and Scolastico *et al.* was confirmed by a completely different approach.^{12,13} Later, it became possible for us to access to a 100.7 MHz NMR spectrometer. The ^{13}C -NMR spectra of the same sample of citrinin (1) enriched with ^{13}C - ^{18}O were measured with 3000 or 4000 Hz spectral width and 32 K data points. One of the spectrum covers the signals of C-1, C-6 and C-8, and the other that of C-3. As it appears in Fig. 3a the shifted signals of C-3, C-6 and C-8 were disturbed by the excess incorporation of labelled acetate, resulting in multiplet peaks due to long range couplings. This problem was readily solved by using diluted labelled acetate to decrease the possibility of simultaneous labelling in the same



Scheme 1. Biosynthesis of citrinin (1).

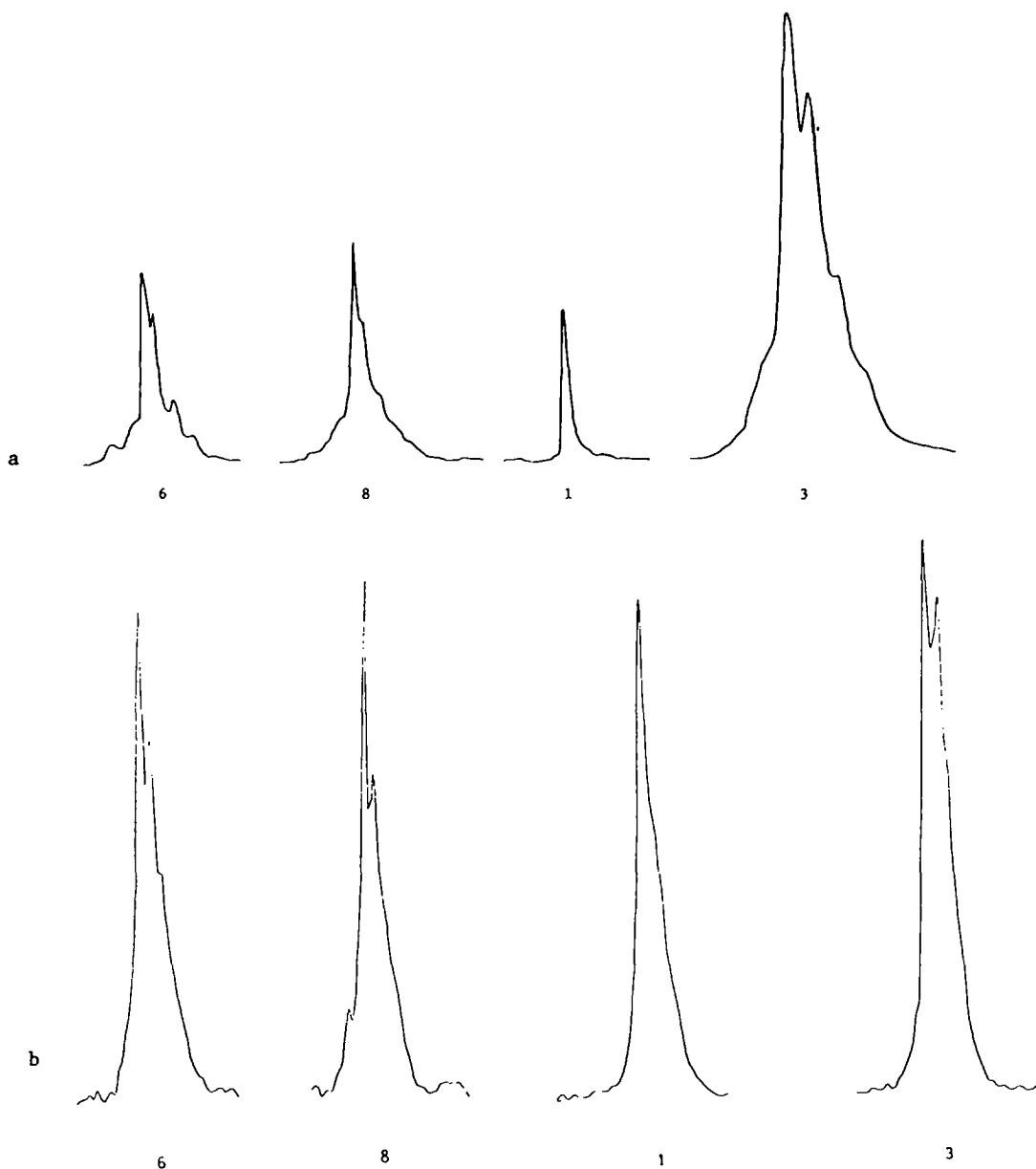


Fig. 2. ^{13}C Signals of citrinin (1) labelled with $[2\text{-}^{13}\text{C}, \text{}^{18}\text{O}]$ -acetate; (a) 25.05 MHz, (b) 50.31 MHz.

citrinin (1) molecule. The same procedure of feeding experiment was repeated except that $[1\text{-}^{13}\text{C}, \text{}^{18}\text{O}]$ -acetate was diluted with three times amount of non-labelled acetate. The ^{13}C signals of citrinin (1) obtained by this feeding experiment are shown in Fig. 3(b), indicating that the multiplet signals due to long range couplings become clear singlets. The values of ^{18}O induced isotope shift of ^{13}C signals are shown in Table 3. During the course of our studies on the biosynthetic application of multiple labelled acetate, Vederas *et al.* extensively studied the application of ^{18}O induced isotope shift in the biosyntheses of polyketides.²⁹⁻³⁵ They also encountered the same ironical problem caused by an excess incorporation of labelled acetate, however they eliminated long range couplings by using spin-echo technique in the measurement of ^{13}C -NMR spectrum.³³

The high incorporation ratios of labelled acetate

into citrinin (1) in *A. terreus*, let us attempt further to investigate the possibility of the utilization of $[1\text{-}^{13}\text{C}, \text{}^{17}\text{O}]$ -acetate as a tracer in polyketide biosynthesis. $[1\text{-}^{13}\text{C}, \text{}^{17}\text{O}]$ -acetate was prepared by a procedure similar to that for the preparation of $[1\text{-}^{13}\text{C}, \text{}^{18}\text{O}]$ -acetate, starting from $[1\text{-}^{13}\text{C}]$ -acetate (90 at. %) and $[\text{}^{17}\text{O}]$ -water (30 at. %). However, $[1\text{-}^{13}\text{C}, \text{}^{17}\text{O}]$ -acetate did not show any ^{13}C signal showing $^{13}\text{C}\text{-}^{17}\text{O}$ coupling. This is because the coupling constant of $^{13}\text{C}\text{-}^{17}\text{O}$ is too small to be used in biosynthetic studies as it was pointed out from theoretical and experimental works.³⁶ On the other hand $[1\text{-}^{13}\text{C}, \text{}^{17}\text{O}]$ -acetate showed a clear ^{17}O signal at 282.5 ppm with natural abundance water as a standard in the ^{17}O -NMR spectrum (12.15 MHz) recorded with 10,000 Hz spectral width and 8 K data points. The incorporation of ^{17}O from this labelled acetate into citrinin (1) could be observed in the ^{17}O -NMR power

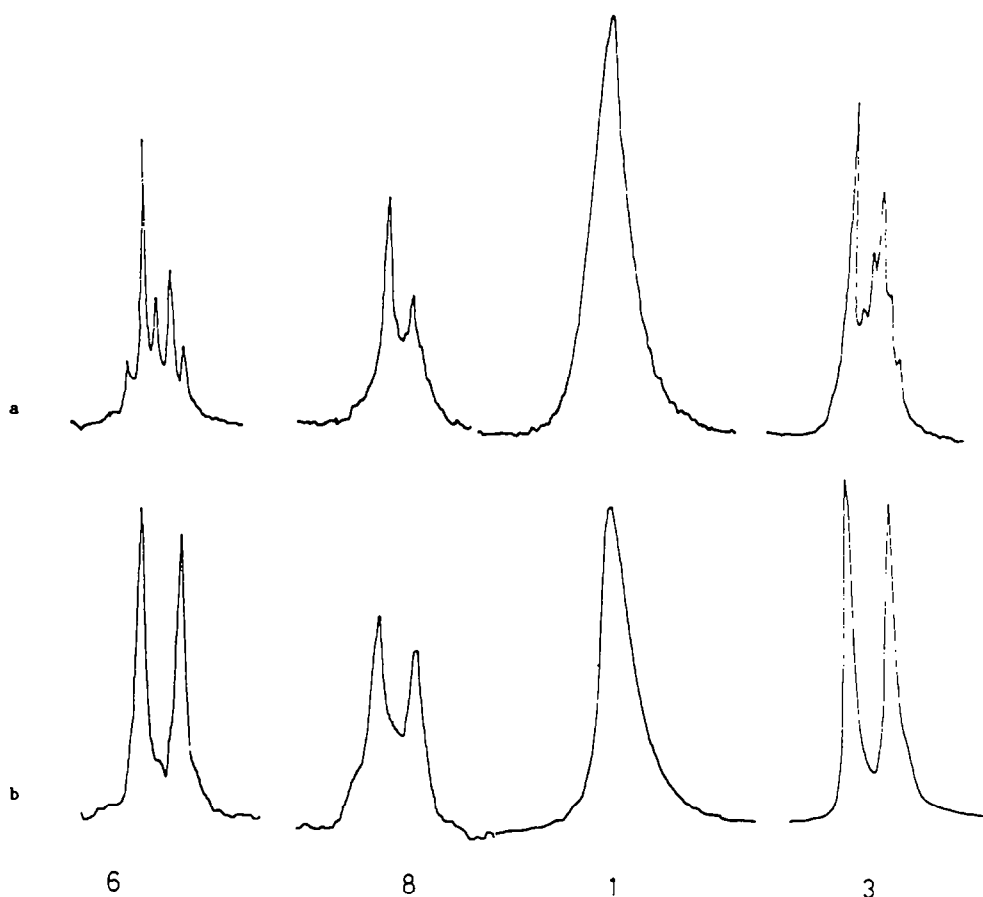


Fig. 3. ^{13}C Signals of citrinin (1) at 100.7 MHz; (a) Labeled with non-diluted $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate, (b) Labeled with $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate diluted with three times amount of non-labelled acetate.

Table 3. ^{13}C - ^{18}O Signals observed in the ^{13}C -NMR spectra of citrinin (1) enriched with $[1-^{13}\text{C}, ^{18}\text{O}_2]$ -acetate

Carbon	Chemical shift (ppm)	^{13}C - ^{18}O Shift(Hz)			^{18}O Retention
		25.05 MHz	50.31 MHz	100.7MHz	
1	162.9	— ^a	— ^a	— ^a	
3	81.8	1.1	2.1	4.2	88 %
6	183.7	1.1	2.1	3.9	89 %
8	177.2	1.1	1.7	3.9	85 %
		(0.38) ^b	(0.06) ^b	(0.25) ^b	

^a ^{13}C - ^{18}O Signal was not detected.

^b Errors in Hz calculated from data points.

spectrum (54.26 MHz) recorded with 40,000 Hz spectral width and 16 K data points and three ^{17}O signals were observed at 148, 179 and 279 ppm as shown in Fig. 4. They are tentatively assigned according to the reported chemical shift data of ^{17}O -NMR.^{37,38} ^{17}O -Chemical shift is very sensitive to a change of electron density on O atom. The chemical shift of C-6 oxygen (279 ppm) is unusually high as a CO and that of C-8 unusually low as a OH. Effects of OH substitution at the *ortho* of the CO groups of benzaldehyde and acetophenone were available and the values of higher field shift caused by forming

chelation with OH groups were 67 and 62 ppm, respectively,^{39,40} indicating that unusual chemical shifts of C-6 and C-8 of citrinin (1) were not merely caused by the presence of strong H-bonds with C-12 carboxyl group. Winter and Zeller reported the ^{17}O chemical shifts of benzoylacetone (4) being 294 and 239 ppm,⁴¹ and accounted for their unusual chemical shifts by a hybrid resonance structure obtained by a low temperature X-ray analysis. In order to clarify the relationship between C-O bond length and ^{17}O -chemical shift in citrinin (1) the authors performed single crystal X-ray analysis of citrinin (1).

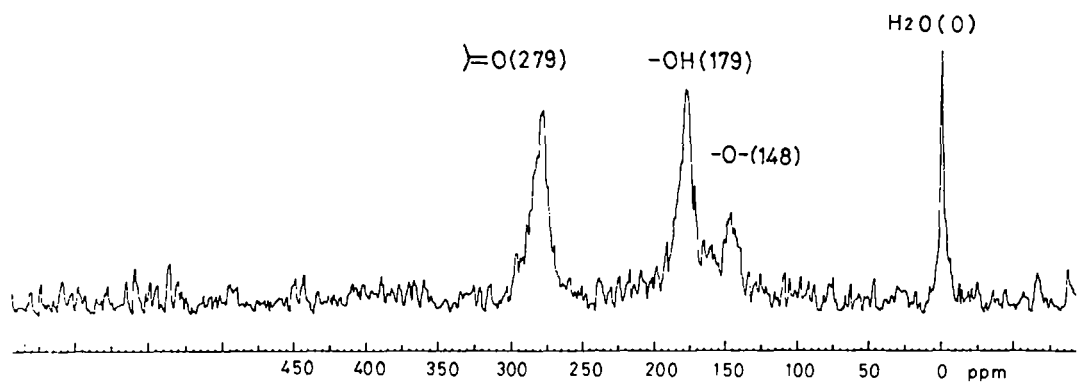


Fig. 4. ^{17}O -Power NMR spectrum (54.26 MHz) of citrinin (1) labelled with $[1-^{13}\text{C}, ^{17}\text{O}]$ -acetate.

The crystals grown in chloroform was suitable for X-ray analysis. The structure was solved by the direct method and the final R value with anisotropic temperature factors for C and O, and isotropic for H was 0.0518. Computer generated drawing and bond lengths are shown in Figs. 5 and 6. A very good correlation was found between ^{17}O chemical shifts and C–O bond lengths of benzoylacetone (4) and

citrinin (1) as it appears in Table 4. The results indicate that the structure of citrinin (1) is not a fixed structure, but the actual structure is visualized as a resonance hybrid somewhat between the two extreme structures (1a and 1b), as benzoylacetone (4) is a resonance hybrid of the two structures (4a and 4b). This also explains unusual ^{17}O chemical shifts of C-6 and C-8. Foregoing results clearly demonstrate that

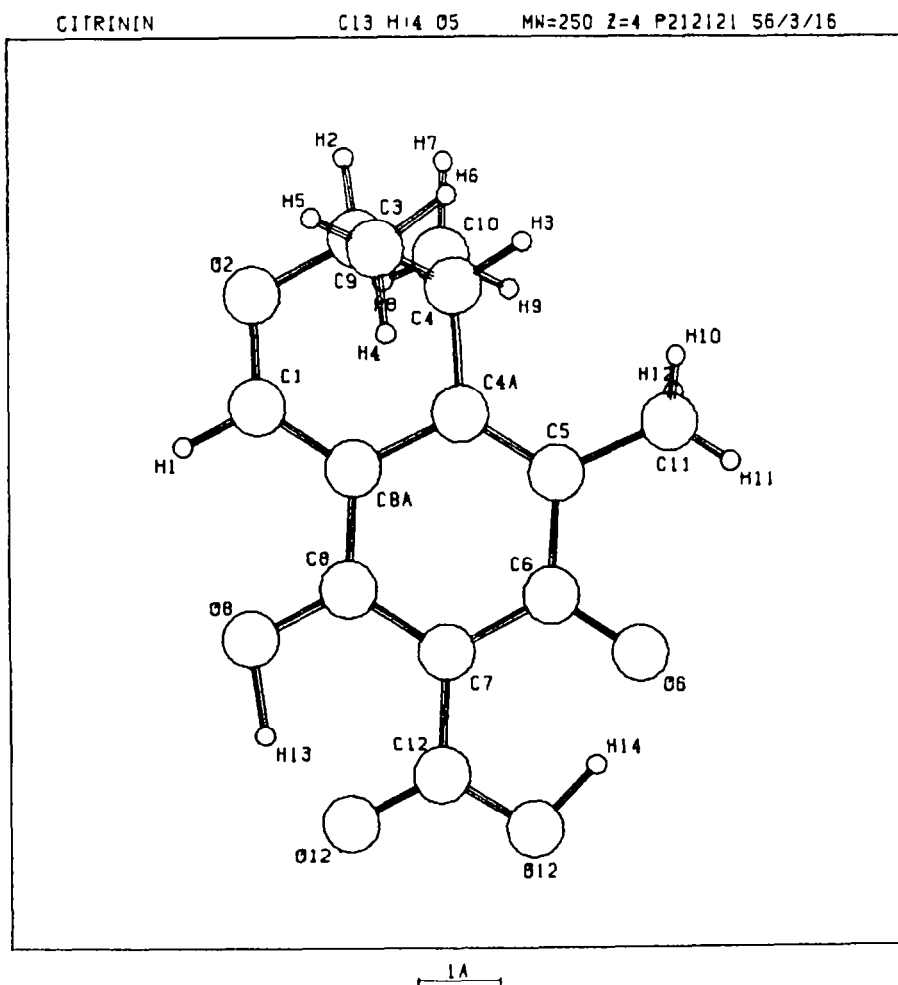


Fig. 5. Computer generated PLUTO drawing of the structure of citrinin (1) obtained by X-ray analysis.

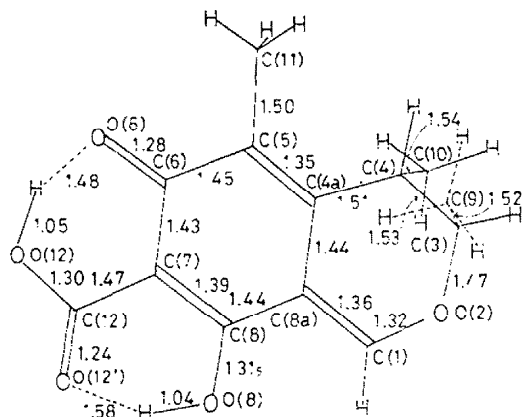


Fig. 6. Bond lengths of citrinin (1).

[1- ^{13}C , ^{17}O]-acetate can be applicable in tracing acetate oxygen in polyketide biosynthesis, though ^{13}C is used only as a monitor for carbon incorporation.

EXPERIMENTAL

Labelled compounds. [1- ^{13}C]-Acetate (90 at. %), [2- ^{13}C]-acetate (90 at. %), [^{18}O]-water (99 at. %) and [^{17}O]-water

(30 at. %) were obtained from B.O.C. Ltd. (Prochem.), London. [1- ^{14}C]-Acetate was purchased from Radio Isotope Center, Japan.

Preparation of multiple labelled acetate. The preparation methods of ^{13}C and ^{18}O or ^{17}O labelled acetate by an exchanging method under an acidic condition are very similar. Here a procedure to prepare [1- ^{13}C , $^{18}\text{O}_2$]-acetate is described as a typical example. [^{18}O]-Water (99 at. %, 0.0625 ml) was saturated with HCl gas at 0° and then [1- ^{13}C]-acetic acid (90 at. %, 0.05 m mole) and [^{18}O]- H_2O (99 at. %, 0.25 ml) were added to HCl saturated [^{18}O]- H_2O . The mixture was sealed in a glass tube and heated at 110° for 24 hr. The mixture was neutralized with NaOH powder and evaporated to dryness under a reduced pressure. The residue was extracted with abs EtOH and [1- ^{13}C , $^{18}\text{O}_2$]-sodium acetate (60 mg) was obtained as a foam on removal of EtOH. In order to measure the isotopic composition, it was converted into *p*-phenylphenacyl acetate. A mixture of [1- ^{13}C , $^{18}\text{O}_2$]-sodium acetate (10.8 mg), *p*-phenylphenacyl bromide (6.9 mg), 18-crown-6-ether (4 mg), benzene (1 ml) and acetonitrile (1 ml) was refluxed for 2 hr. After usual workup, *p*-phenylphenacyl acetate was recrystallized from EtOH to give colourless crystals of m.p. 106–107° (27.2 mg). The high resolution mass spectrum revealed that the labelled acetate consists of 74.6% [1- ^{13}C , $^{18}\text{O}_2$], 8.4% [$^{18}\text{O}_2$] and 16.4% [1- ^{13}C , ^{18}O]-acetate.

Incorporation experiment. *Aspergillus terreus* ATCC 24839, maintained on potato dextrose agar slant, was inoculated in a modified Czapek-Dox medium (150 ml/flask)

Table 4. ^{13}C and ^{17}O -NMR chemical shifts and C-O bond lengths

	Chemical shift (ppm)		C-O Bond length (Å)
	^{13}C	^{17}O	
Citrinin(1)			
C-1	162.9	148	1.32
C-2	183.7	274	1.28
C-8	177.2	179	1.315
Benzoylacetone(4) ^a			
C-1	183	239	1.292
C-3	193	294	1.278

^a The data were reported by Winter and Zeller⁴¹.

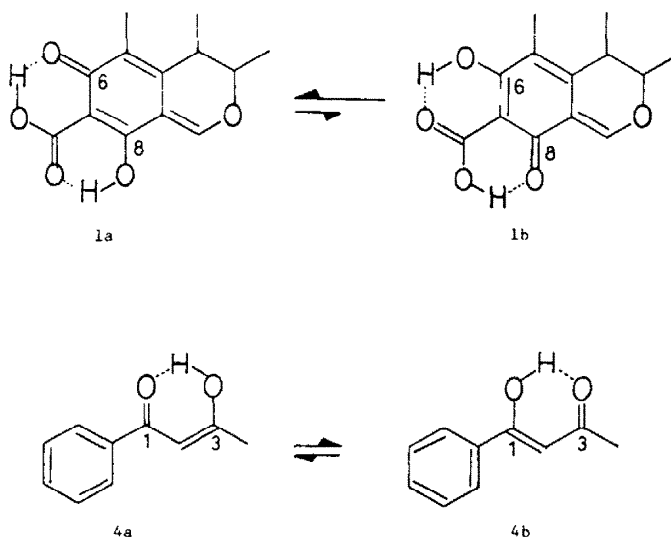


Fig. 7. Resonance formulae of citrinin (1) and benzoylacetone (4).

Table 5. Fractional atomic coordinates and anisotropic thermal parameters for non-hydrogen atoms, $\times 10^4$. Fractional atom coordinates, $\times 10^3$, and isotropic thermal parameters for hydrogen atoms, $\times 10^2$

ATOM	X	Y	Z	U ₁₁	U ₂₂	U ₃₃	U ₁₂	U ₁₃	U ₂₃
C(1)	8587(2)	7791(2)	7219(4)	393(11)	478(12)	585(16)	-63(9)	-56(12)	30(12)
O(2)	8275(2)	8724(1)	7005(3)	571(11)	386(8)	720(13)	-117(8)	-85(11)	33(8)
C(3)	7110(3)	8893(2)	6656(4)	590(15)	394(11)	518(15)	63(12)	-43(14)	-8(10)
C(4)	6406(2)	8223(2)	7860(4)	470(12)	482(12)	533(12)	128(10)	27(12)	3(12)
C(4A)	6736(2)	7152(2)	7614(4)	339(9)	420(10)	405(12)	51(9)	-12(10)	7(10)
C(5)	6034(2)	6376(2)	7691(4)	310(9)	571(14)	627(16)	-28(9)	17(11)	19(12)
C(6)	6474(2)	5373(2)	7604(4)	488(11)	481(12)	566(16)	-13(11)	-39(13)	42(12)
C(7)	7522(2)	5205(2)	7507(4)	516(11)	373(11)	453(13)	40(10)	3(12)	25(11)
C(8)	8330(2)	6012(2)	7450(4)	349(10)	470(11)	456(13)	44(9)	4(10)	-2(11)
C(8A)	7998(2)	7006(2)	7415(3)	341(9)	374(9)	401(13)	-9(8)	4(10)	-25(10)
C(9)	6997(4)	8752(3)	4625(5)	888(24)	559(16)	506(15)	97(17)	-116(17)	83(13)
C(10)	6500(4)	8515(3)	9895(5)	1060(32)	620(18)	581(18)	215(20)	174(20)	-68(16)
C(11)	4817(3)	6474(3)	7931(7)	310(10)	935(26)	1355(39)	-28(15)	48(20)	94(28)
C(12)	9059(4)	4188(2)	7493(5)	821(20)	413(12)	632(18)	79(13)	18(8)	0(14)
O(6)	5815(2)	4634(2)	7630(5)	661(14)	614(13)	1156(23)	-310(11)	1(15)	02(16)
O(8)	9393(2)	5878(2)	7356(4)	338(8)	628(12)	1053(19)	11(8)	15(12)	-29(13)
O(12)	7365(3)	3462(2)	7501(4)	1112(21)	367(9)	1098(21)	-34(12)	-18(21)	44(14)
O(12A)	9059(3)	4039(2)	7466(5)	835(17)	577(13)	1081(21)	296(12)	-71(17)	-8(14)
H(C1)	941(3)	767(2)	724(5)	6(1)					
H(C3)	690(3)	959(2)	695(5)	5(1)					
H(C4)	560(3)	859(3)	726(5)	7(1)					
H(C9)	705(4)	801(3)	416(6)	8(1)					
H'(C9)	737(4)	918(4)	406(6)	12(2)					
H'(C9)	606(4)	899(3)	431(6)	9(1)					
H(C10)	619(4)	928(4)	1009(6)	10(2)					
H'(C10)	712(5)	848(5)	1013(8)	12(2)					
H'(C10)	500(4)	805(4)	1065(6)	9(1)					
H'(C11)	452(4)	700(3)	724(7)	10(1)					
H'(C11)	437(5)	598(4)	789(8)	13(2)					
H'(C11)	471(6)	671(5)	874(9)	15(2)					
H'(C11)	471(6)	502(3)	721(9)	13(2)					
H(O8)	956(5)	383(3)	781(6)	7(1)					

Temperature factor T is the form of

$$T = \text{EXP}(-2\text{PAI}^2(U_{11}\text{HHA}^*A + U_{22}\text{KKB}^*B + U_{33}\text{LLV}^*C + 2U_{12}\text{HKA}^*B + 2U_{13}\text{HHA}^*C + 2U_{23}\text{KKB}^*C))$$

containing glucose 50 g, NaNO₃ 2.0 g, K₂HPO₄ 1.0 g, KCl 0.5 g, MgSO₄ 7H₂O 0.5 g, FeSO₄ 7H₂O 0.01 g, ZnSO₄ 7H₂O 0.01 g, CuSO₄ 5H₂O 0.005 g, malt extract 0.5 g and yeast extract 0.2 g in 1 l. H₂O and grown stationarily at 30°. Only a typical feeding experiment is described here. [1-¹³C, ¹⁸O₂]-Na acetate (25 mg), non-labelled Na acetate and [1-¹⁴C]-Na acetate (10 µCi) was dissolved in 5 ml H₂O and administered to the culture (150 ml medium) each 0.5 ml for 10 days starting from the 9th day after inoculation. The culture was harvested on the 21st day. The culture medium was acidified (pH 3) with 4-N HCl and extracted with AcOEt (150 ml × 2) and AcOEt layer was further extracted with 5% NaCO₃ (150 ml × 2). Alkaline aqueous layer was then acidified with 6N-HCl to pH 3 to liberate free citrinin (I) and extracted with AcOEt. Organic layer was washed thoroughly with H₂O, dried over Na₂SO₄ and evaporated to dryness. The residue was dissolved in CHCl₃ and chromatographed on acidified silica gel (impregnated with 0.5 N oxalic acid). A yellow band eluted from the column gave crude citrinin (I) (147 mg). Upon recrystallization from AcOEt gave golden yellow needles of m.p. 161–162°.

Mass and NMR spectra. Mass spectrum was recorded on a JEOL DX-200 equipped with computer data processing system. ¹³C-NMR spectra was recorded on a JEOL FX-100, Varian XL-200 or JEOL FX-400. ¹⁷O-NMR spectra was recorded on a JEOL FX-90Q or FX-400.

X-Ray analysis of citrinin (I). The crystals of I grown in CHCl₃ was found suitable for X-ray analysis. The crystal is orthorhombic, space group P2₁2₁2, with four molecules in a cell of dimension, a = 12.238(6), b = 13.4521(7), c = 7.3000(4) Å, Dx = 1.38 cm⁻³ and V = 1201.8 Å³. A total of 1336 reflections was recorded within the θ range of 3–60°. The structure was solved by the direct method (MULTAN) and refined by the block-diagonal least-squares (BLS). An R value was 0.0522 for 1336 reflections including anisotropic temp factors for 13 carbon and 5 oxygen atoms, and isotropic for 13 H atoms. Further refinement with 8 times BLS and one of methyl H atom not found before was placed on the calculated position to give a final R value of 0.0518.

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