New Insights into Polyketide Metabolism; the Use of Protium as a Tracer in the Biosynthesis of Citrinin by *Penicillium citrinum* †

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Evidence concerning the origin of the hydrogen atoms in the polyketide antibiotic citrinin has been obtained by a novel technique in which the metabolite is produced from protium-labelled carbon sources on a medium based on D_2O.

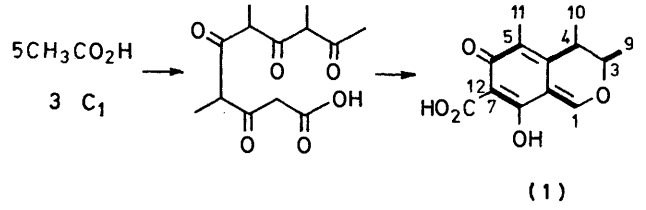
THE problem of tracing hydrogen in biosynthesis has attracted much interest, and many isotopic methods for elucidating pathways have been devised. Classically, tritium was used as a label,[1] often in conjuction with a ^{14}C standard, and was detected by scintillation counting. The major problems with this technique are that extensive degradation is required to determine the specificity of labelling, and that the multiplicity of label at a given site can only be inferred at best. The use of deuterium as a label with detection by ^2H n.m.r.[2] overcomes the first of these difficulties but the second remains a limitation when dilution has occurred. Deuterium can also be detected by mass spectrometry[3] but interpretation of data is difficult, requiring complicated statistical analysis.

In order to overcome these problems deuterium has been detected indirectly *via* its coupling to ^{13}C in the ^{13}C n.m.r. spectrum.[4-6] However, the direct bonding of deuterium increases the relaxation times of carbon atoms relative to those of the protonated species. Furthermore, the nuclear Overhauser effect which enhances the intensity of signals due to carbons bonded to protium in the proton-noise-decoupled spectrum is not observed for deuteriated carbon atoms. Consequently, although deuterium has been monitored in a number of cases *via* ^{13}C n.m.r spectra, observation of ^{13}C–D signals is difficult when the associated ^{13}C–H signals are relatively strong or when sample sizes are small.

We have successfully overcome this difficulty by reversing the roles of the two hydrogen isotopes, *i.e.* using deuterium (in D_2O) as the background isotope and protium as the tracer in the precursor under investigation.

RESULTS

The polyketide antibiotic citrinin (1) was chosen as a case study. Early work using ^{14}C as a tracer[7] established that the carbon skeleton of citrinin is biosynthesised by *P. citrinum* and a number of other fungi from five acetate and three C_1 units (Scheme). Evidence concerning the identity of advanced intermediates has been sought from mutant studies[8] and by incorporation experiments.[9] In the latter it was found that a radioactive label was incorporated specifically, though to a very small extent (0.005%), from the isocoumarin (2) but not from the related (3).

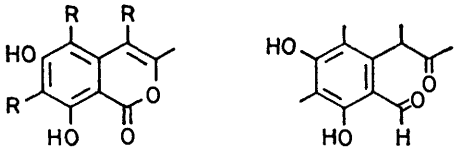


(1)

SCHEME

† Preliminary communication, J. Barber and J. Staunton, *J.C.S. Chem. Comm.*, 1979, 1098

Because of its very low incorporation we suspected that (2) might not be a true precursor of citrinin. Clearly, since (1) has a proton at C(4) and (2) has not, experiments to



(2) R = Me
(3) R = H

(4)

determine the origin of the C(4) proton of citrinin should resolve this uncertainty: if hydrogen from acetate survives at C(4) in citrinin then (2) cannot be an obligatory precursor. We were also concerned to discern the origin of the other hydrogen atoms in the citrinin molecule.

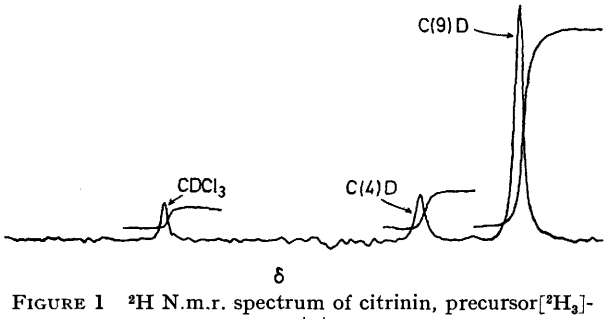


FIGURE 1 ^2H N.m.r. spectrum of citrinin, precursor[$^2H_3$]-acetate

The simplest technique now available to study the fate of hydrogen atoms from acetate is to administer CD_3CO_2Na and observe deuteriation in the resulting citrinin by means of deuterium n.m.r. This experiment was performed and the resulting ^2H n.m.r. spectrum showed two signals which, by analogy with the chemical shifts in the ^1H n.m.r. spectrum, could be shown to be due to retention of deuterium at C(4) and C(9) (Figure 1). The signal due to deuterium at C(9) integrates to about 5 times that due to C(4). Clearly there is extensive loss of deuterium at C(4) compared with C(9) but this experiment proves that deuterium can be retained at that position. If the isocoumarin (2) had been a precursor it would be expected that C(4) would derive its hydrogen from the medium and would therefore be devoid of deuterium. However, there remains the slight possibility in the controlled environment of the living cell that deuterium washed out from one intermediate

might be reincorporated into subsequent intermediates faster than it is exchanged with the medium of the cell at large. The other limitation of this sort of experiment, when, as seems to be the case in polyketide biosynthesis, there is extensive loss of label by exchange, is that it gives only information concerning the average enrichment of the site, and does not reveal the multiplicity of labelling in individual molecules.

This extra insight has in some cases been gained by the use of an internal ^{13}C label. In the biosynthesis of terrein,[10] for example, it was possible to identify a chain starter methyl group by administering $^{13}CD_3CO_2Na$ and observing multiplets in the proton-noise-decoupled ^{13}C spectrum and a peak due to a wholly deuteriated methyl group in the deuterium-decoupled spectrum. When this technique was applied to citrinin an enrichment of ^{13}C over natural abundance was obtained at the expected sites (2%). However, it was not possible to detect multiplets due to deuterium coupling in signals for C(4) or C(9). In the light of the dueterium n.m.r. study described above, these must be present but presumably they are either lost in the noise or are obscured by the adjacent signal from protonated carbons.

This failure illustrates the limitations of this technique. Replacement of protons by deuterons at a ^{13}C nucleus causes a serious loss of signal intensity and hence there is an inherent bias against deuterium detection in this type of experiment. We were therefore attracted to the possibility of reversing the roles of the isotopes ^1H and ^2H, and we now describe a technique which allows this to be achieved.

A complete reversal would involve the use of a fully deuteriated carbon source (in this case glucose) dissolved in D_2O. Appropriately labelled acetate ($^{13}CH_3CO_2Na$) would then be administered. The requisite glucose would, however, be prohibitively expensive for routine use. We have therefore devised an alternative strategy in which normal unenriched glucose is used as the major carbon source. The endogenous acetate produced under these conditions could contain as many as two protons at C(1) but none of the molecules should contain a CH_3 group. Hence, provided it is possible to distinguish carbon atoms from this source from those derived from the administered acetate, a controlled study of hydrogen isotope retention is possible. This was achieved by using $^{13}CH_3{}^{13}CO_2Na$. The signals due to glucose-derived carbon atoms appear in the proton-noise-decoupled ^{13}C n.m.r. spectrum of citrinin as singlets (ignoring any deuterium coupling). The acetate-derived carbon atoms give rise to doublets (due to ^{13}C–^{13}C coupling), which flank the natural-abundance singlets. Apart from the saving in cost by using ^1H-glucose there is an added bonus in that information can be gained concerning the origins of all the hydrogen atoms attached to carbon in the molecule under study.

In the current experiment *Pencillium citrinum* was grown from spores on a Czapek-Dox medium based on D_2O. The culture grew sluggishly, but after 14 days citrinin production had begun. The medium was replaced by a glucose-depleted medium (also based on D_2O) and [1,2-$^{13}C_2$]acetate was administered in equal daily doses for ten days. After a further ten days the citrinin was harvested. A control experiment in which [1,2-$^{13}C_2$]acetate was administered to a culture of *P. citrinum* grown on an H_2O-based medium was carried out similarly. Proton and ^{13}C (both in the absence and in the presence of a relaxation agent) n.m.r. spectra were taken of both samples. The ^1H n.m.r. spectrum of unenriched citrinin is given in Table 1.

TABLE 1

^1H n.m.r. spectrum of unenriched citrinin ($CDCl_3$)

δ/p.p.m.	Integral (multiplicity)	J/Hz	Position
1.23	3H (d)	7.3	C(10)H_3
1.35	3H (d)	6.8	C(9)H_3
2.02	3H (s)		C(11)H_3
2.99	1H (q)	7.3	C(4)H
4.78	1H (q)	6.8	C(3)H
8.24	1H (s)		C(1)H
15.09	1H (s)		C(8)OH
15.86	1H (s)		C(12)O_2H

The ^1H n.m.r. spectrum of citrinin derived from the D_2O experiment is shown in Figure 2. The major differences are

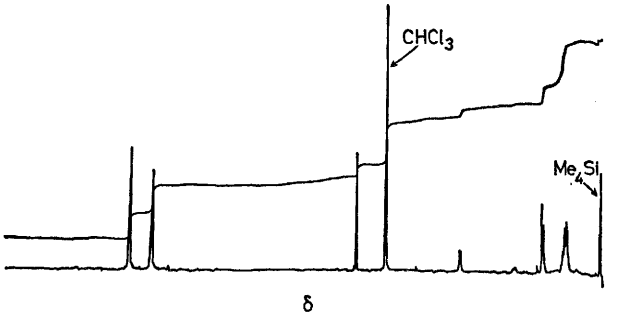


FIGURE 2 ^1H N.m.r. spectrum of citrinin produced on D_2O

as follows: (*a*) the signals due to protons bonded to C(9) and C(10) have collapsed to complex overlapping multiplets which integrate to a total of 1.5H (the OH protons, which are completely protonated during work-up, are taken as standards); (*b*) the signals due to protons at C(11) comprise a 1 : 1 : 1 triplet with a singlet at lower field and smaller satellites at higher field (the total integral is 0.7H); (*c*) the signals due to protons at C(4) and C(3) have collapsed to broad multiplets integrating to 0.19 and 0.34H respectively; and (*d*) the singlet due to the proton at C(1) is unchanged in appearance but integrates to only 0.45H.

The natural abundance ^{13}C n.m.r. spectrum of citrinin shows 13 peaks (Table 2). In the control experiment in which [1,2-$^{13}C_2$]acetate was administered to an H_2O-based culture of *P. citrinum*, the citrinin produced showed three unchanged signals, those due to C(10), C(11), and C(12) when the spectrum is run in the presence of chromium tris-(acetonylacetonate). The other signals consisted of a singlet flanked by a doublet and the coupling constants con-

TABLE 2

^{13}C Chemical shifts and ^{13}C–^{13}C coupling constants of citrinin (in $CDCl_3$)

Carbon	δ/p.p.m.	J(C–C)/Hz
1	161.95	69.6
3	80.91	37.8
4	33.72	40.9
4a	139.17	40.9
5†	99.36	63.6
6‡	176.15	63.6
7†	122.01	56.9
8‡	182.73	56.9
8a	106.44	69.6
9	17.56	37.8
10	8.72	
11	17.83	
12	173.48	

†‡ Signals may be interchanged.

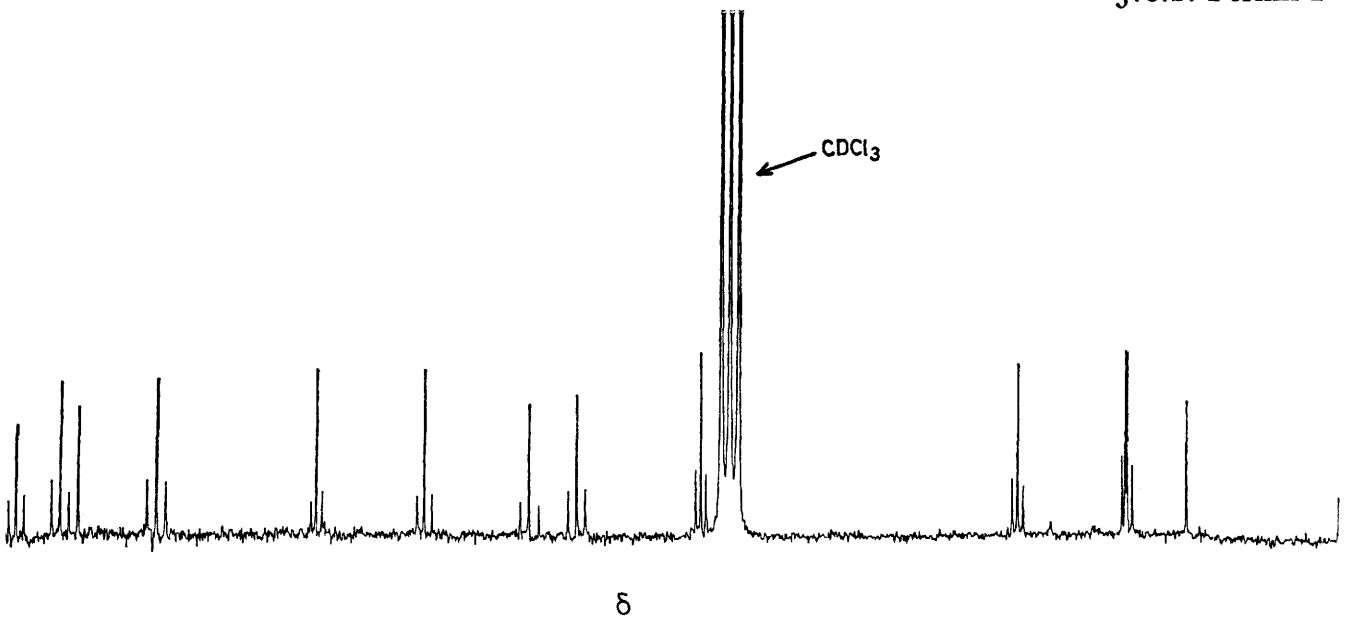


FIGURE 3 ^{13}C N.m.r. spectrum of citrinin produced on H_2O, precursor[1,2-$^{13}C_2$]acetate

firmed that citrinin is biosynthesised by head-to-tail linkage of five acetate units. This spectrum is shown in Figure 3.

The ^{13}C spectrum of citrinin from the D_2O experiment run under similar conditions with a relaxation agent is shown in Figure 4. Although some of the signals are profoundly different from the previous spectrum, a number, especially those derived from carbon nuclei remote from hydrogen nuclei are essentially similar in appearance. This shows that the conditions of the D_2O experiment do not seriously disturb the carbon metabolism of the fungus. The altered signals are those due to C(1), (3), (4), (9), (10), and (11) which appear as complex multiplets and C(8a) in which each of the three normal lines is accompanied by an additional line slightly upfield.

In order to enhance the intensity of the signals due to protonated carbon atoms relative to the rest of the spectrum, the ^{13}C spectra were also run in the absence of any relaxation agent. Predictably the shapes of the signals of the control were unchanged, the only difference between this spectrum and Figure 3 being that signals due to the quaternary carbon atoms did not appear. The corresponding spectrum for the D_2O experiment showed important differences, however. This spectrum is shown in Figure 5.

The signal due to C(10) appears only as a broad hump made up of unresolved multiplets, and that due to C(11) is obscured by the C(9) signal. The signals due to C(1) and C(3) revert to the pattern of the control spectrum, while those due to C(4) and C(9) appear as strong doublets with weak associated singlets.

In total these spectra provide information about the

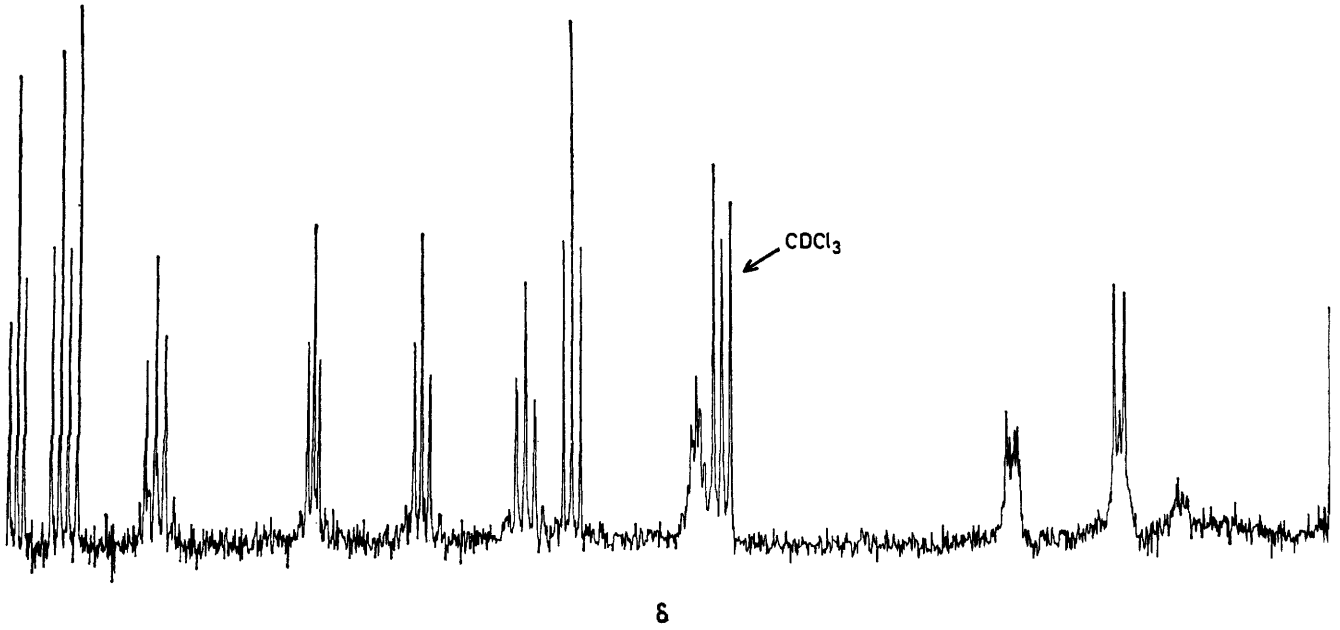


FIGURE 4 ^{13}C N.m.r. spectrum of citrinin produced on D_2O, precursor[1,2-$^{13}C_2$]acetate

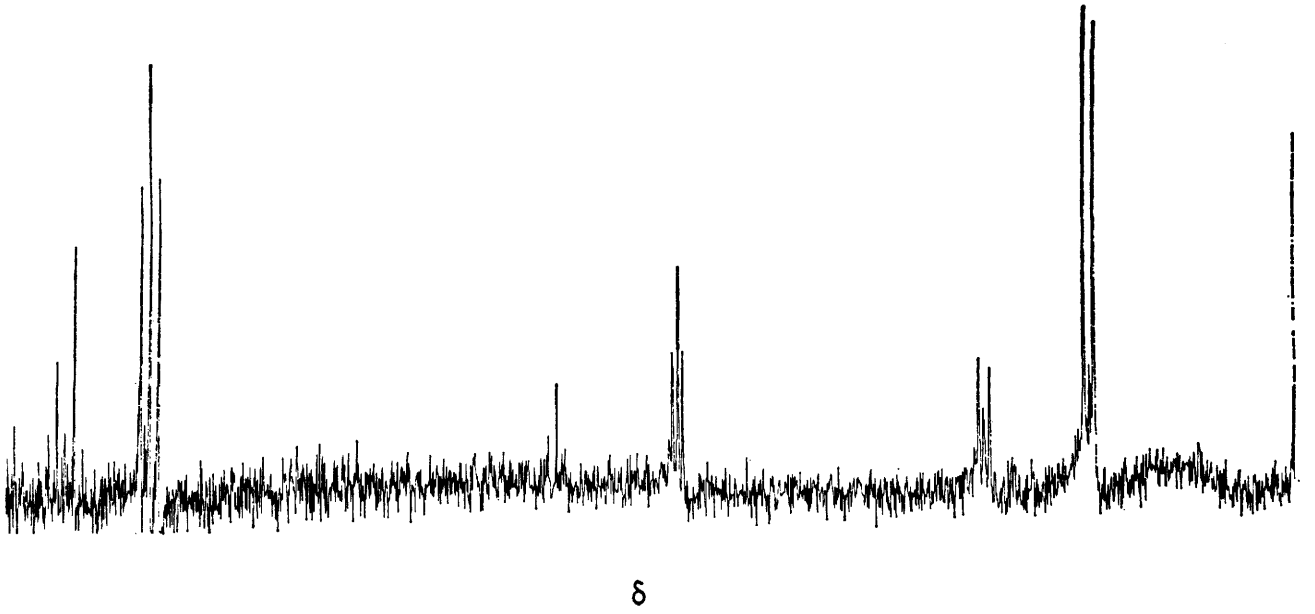


FIGURE 5 ^{13}C N.m.r. spectrum of citrinin produced on D_2O, precursor[1,2-$^{13}C_2$]acetate (no added relaxation agent)

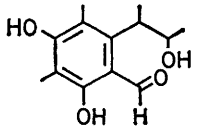
origin of all the hydrogen atoms bonded to carbon, as explained in the Discussion section.

DISCUSSION

The presence of a strong doublet corresponding to C(9) in the ^{13}C spectrum indicates that intact $^{13}CH_3$–^{13}C residues from acetate are incorporated at this position. This result confirms that (C9) is a part of the chain-starter unit. This is a sensitive method of detecting chain-starter methyl groups, and provides an attractive alternative to established methods.[10-12]

A doublet with weak associated signals is also given by C(4). This, together with the ^1H n.m.r. spectrum, confirms that protons can be retained at this site, supporting the conclusions gained from the ^2H n.m.r. study that (2) is not an obligatory precursor to citrinin. However, this experiment additionally rules out the possibility that a proton rather than a deuteron has been unexpectedly added at a late stage of the biosynthesis or that exchange had occurred during work up. The very strong doublet in the ^{13}C n.m.r. associated with a weak singlet shows that carbon atoms derived from the administered acetate are protonated to a much greater extent than those derived from glucose. If protonation were to occur late in the biosynthesis there would not be this discrimination between carbons from the alternative sources. C(1) and C(3) are both carboxy-derived and so, as expected, the ^1H : ^2H ratio at these positions is the same for carbon atoms derived from administered acetate as for those derived from glucose. The hydrogen must be derived from an external source, presumably from a cellular coenzyme. The obvious candidates for this role are the flavin and the nicotinamide coenzymes. However, the reducing hydrogen atoms of the flavin coenzymes are readily exchanged with the medium, whereas those of nicotinamide are not. This suggests that C(1) and C(3) are reduced by enzymes dependent on nicotinamide coenzymes, the reducing hydrogen being derived ultimately partly from the medium and partly from the C–H bonds of glucose.

A potentially very interesting feature of these positions is shown in the integral measured by a series of ^1H n.m.r. spectra, in which the C(1)-H integrates to 0.48 ± 0.05 and C(3)-*H* to only 0.35 ± 0.05. The integrals in a pulsed natural-abundance spectrum run under similar conditions were 1 ± 0.05 for both C(1)-*H* and C(3)-*H*. This differential incorporation would not be expected if both reductions were carried out in rapid succession in the same part of the cell. It is hoped that feeding experiments with (4) and (5) will throw further light on the timing of these reductions in the biosynthesis of citrinin.



(5)

Finally, an insight was gained into C_1 metabolism. The two methyl groups derived from the C_1 pool, *i.e.* C(10) and C(11), both show substantial deuteriation in the ^{13}C n.m.r. spectrum taken without a relaxation agent. Thus C(10) gives rise to a broad envelope. On addition of chromium tris(acetonylacetonate) a complex multiplet is revealed. The signal due to C(11) is obscured by the C(9) signal. In the ^1H n.m.r. spectrum the signal due to C(11) can be discerned, and it appears as a singlet superimposed on a strong 1 : 1 : 1 triplet with some weaker satellite signals. However, the integral is only 0.7H. Thus, although the presence of all three protonated species (CH_3, CH_2D, and CHD_2) is indicated, CD_3 must be present as a major species. Hence, it seems likely that the methyl groups are derived from glucose mainly *via*

formic acid, which is reduced by nicotinamide to the methyl group of *S*-adenosylmethionine.

In conclusion, therefore, this technique provides information concerning the origin of all the hydrogen atoms attached to the carbon skeleton of citrinin, not merely those derived from acetate. It is considerably more sensitive than the existing method using $^{13}CD_3CO_2H$ and should prove to be of value for investigating the biosynthesis of terpenes and steroids, as well as polyketides.

EXPERIMENTAL

Penicillium citrinum was grown from spores on a Czapek-Dox medium based on D_2O (99.8%, 400 ml) at a constant temperature of 27 °C. When the mycelium covered the medium and citrinin production had begun (after 14 days) the mycelium was refloated on fresh medium, also based on D_2O (99.8%, 120 ml) and containing a reduced concentration of glucose. Administration of sodium [1,2-$^{13}C_2$]acetate (100 mg, ^{13}C 93%, 92%) mixed with unenriched sodium acetate (75 mg) dissolved in D_2O (99.8%, 10 ml) was begun on the day the medium was changed, 1 ml being syringed through the mycelium daily for each of the next 10 days. After a further 10 days the citrinin was harvested as follows.

The medium was separated from the mycelium by filtration through a glass wool plug and was then reduced to a quarter volume by evaporation. It was acidified to pH 2 with concentrated hydrochloric acid and then extracted into ethyl acetate (3 × 20 ml). (Emulsions formed during this stage were separated with phase separator paper.) The combined organic layers were extracted into saturated sodium hydrogencarbonate solution (6 × 20 ml) and the combined extracts acidified to pH 2 (with concentrated hydrochloric acid) and re-extracted with ethyl acetate (4 × 20 ml). The ethyl acetate solution was washed with brine, dried, and evaporated to give yellow crystals of citrinin, which were recrystallised from 95% ethanol (yield 18 mg).

In the control experiment *P. citrinum* was cultured similarly on an H_2O-based medium. The mycelium grew more strongly and the medium was changed on the eighth day of growth. Citrinin was harvested after a total growth time of 31 days. (Yield 21 mg.)

^{13}C N.m.r. spectra were run on a Varian XL100A spectrometer using a 5-mm insert and with ^{13}C-depleted deuteriochloroform (Prochem) as solvent; ^1H n.m.r. spectra were run on the same instrument with deuteriochloroform as solvent. The ^2H n.m.r. spectra were run on a Varian XL200 spectrometer using unenriched chloroform as solvent and natural abundance deuteriochloroform as internal standard.

Standard Czapek-Dox Medium.—Glucose (20 g), sodium nitrate (800 mg), potassium dihydrogenphosphate (400 mg), potassium chloride (200 mg), $MgSO_4 \cdot 7H_2O$ (200 mg), $FeSO_4 \cdot 7H_2O$ (trace), $ZnSO_4 \cdot 7H_2O$ (trace), $CuSO_4 \cdot 5H_2O$ (trace), and yeast extract (trace), made up to 400 ml with H_2O or D_2O.

Replacement medium. Glucose (3 g), sodium nitrate (240 mg), potassium dihydrogenphosphate (120 mg), potassium chloride (60 mg), $MgSO_4 \cdot 7H_2O$ (60 mg), $FeSO_4 \cdot 7H_2O$ (trace), $ZnSO_4 \cdot 7H_2O$ (trace), $CuSO_4 \cdot 5H_2O$ (trace), and yeast extract (trace), made up to 120 ml with H_2O or D_2O.

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