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THE BIOSYNTHESIS OF TRICHOTHECIN FROM ACETATE-[1,2-$^{13}C_2$]*

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Key Word Index—*Trichothecium roseum*; Mucedinaceae; trichothecin; ^{13}C NMR spectroscopy; biosynthesis; sesquiterpenoid.

Abstract—The coupling pattern of trichothecin biosynthesized from acetate-[1,2-$^{13}C_2$] is in accord with previous enrichment studies. Multiple labelling was observed. Exogenous acetate has been shown to inhibit the utilization of glucose and the incorporation of radioactivity from pyruvate-[2-^{14}C] and citrate-[1,5-^{14}C] into the metabolites. Two pairs of ^{13}C NMR assignments are interchanged.

INTRODUCTION

The trichothecene mycotoxins have recently attracted considerable attention in terms of their structure, biosynthesis and biological activity [1]. The fungus, *Trichothecium roseum* produces [2] trichothecin (**3**) and trichothecolone (**4**). In our ealier biosynthetic studies with the fungus, we assigned [3] the ^{13}C NMR resonances of the trichothecenes and used the results to define the labelling pattern of trichothecolone biosynthesized from mevalonate-[2-^{13}C]. However, the enrichment was low [4] and recently some assignments of the ^{13}C resonances have been questioned [5–7]. We have therefore returned to this problem and studied the incorporation of acetate-[1,2-$^{13}C_2$] into the metabolites. By measuring the ^{13}C:^{13}C coupling constants, it is possible to link pairs of carbon atoms, and thus, in conjunction with the other parameters of chemical shift and multiplicity in the SFORD spectrum, to clarify assignments. Secondly the two foldings of farnesyl pyrophosphate (**1**) and (**2**), which it was our initial objective to distinguish, generate different coupling patterns in the metabolites when they are biosynthesized from acetate-[1,2-$^{13}C_2$]. Such measurements are not open to the objections associated with a low enrichment, and by involving atoms other than those originating from C-2 of mevalonate, should complement our previous studies.

RESULTS AND DISCUSSION

Prior to the labelling experiments, a number of fermentation parameters were examined in order to minimize the dilution of the label. In the strain (CMI 50660) utilized for this work, trichothecin production does not occur (Fig. 1) until the idiophase is reached. The maximum incorporation (Fig. 1) of acetate-[2-^{14}C] over a 48 hr incubation period, was during this phase. The minimum dilution is clearly early in this stage and hence an incubation period compatable with the production of a 'workable' amount of material and an acceptable dilution lay from day 5 of the fermentation. The effect of increasing acetate concentration at different sugar levels, on the yield of trichothecin and the incorporation, was also examined. High concentrations of acetate appear to be inhibitory. Hence the labelled material, 98% enriched at both carbon atoms, was fed at a concentration of 50 mg/100 ml on day 5 and the trichothecenes were isolated 48 hr later.

The ^{13}C NMR spectra of the labelled trichothecin and trichothecolone were considerably more complex than we had anticipated. In particular there was coupling between acetate units as well as within acetate units, and in the case of C-3 and C-4, and C-7 and C-8, the coupling was detectable between isoprene units. Thus a substantial number of trichothecin and trichothecolone molecules had been formed containing more than one $^{13}C_2$ unit. Examination of the ion-intensities in the MS (Table 1) confirmed this. Approximately 30% of the added acetate-[$^{13}C_2$] was recovered from the broth at the end of the experiment. Examination of its ^{13}C NMR spectrum showed no change in the ratio of the coupled doublet to the singlet signal, arising from uncoupled material. This recovery, apart from enabling acetate-[$^{13}C_2$] to be re-used, showed that the exogenous acetate had not fully equilibrated with endogenous material.

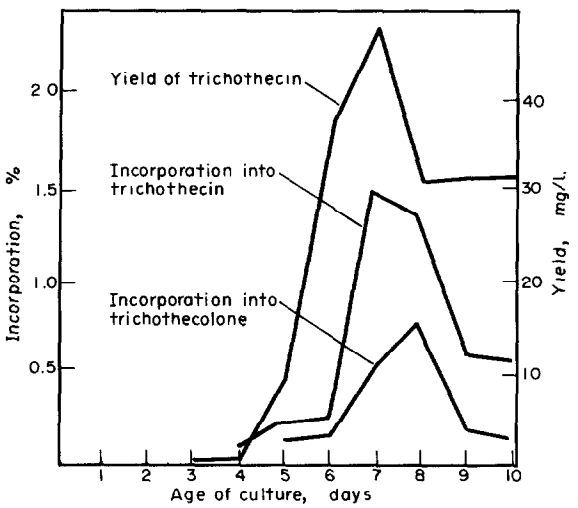


Fig. 1. The yield and the incorporation of sodium acetate-[2-^{14}C] into the metabolites of *T. roseum*.

* Part 20 in the series 'Studies in Terpenoid Biosynthesis'. For part 19 see Dockerill, B. and Hanson, J. R. (1977) *J. Chem. Soc. Perkin I*, 324.

Table 1. Relative ion intensities in the mass spectrum of trichothecin

Ion (*m/e*)	246	247	248	250
Unlabelled material	100	18.7	2.7	0.75
^{13}C enriched trichothecin. Expt. 1.	100	20.5	7.6	3.80
^{13}C enriched trichothecin. Expt. 2.	100	19.7	4.0	0.74

The formation of multiply-labelled trichothecin molecules suggested that the exogenous acetate had exerted a regulatory control over normal acetate biosynthesis. We therefore examined the effect of acetate on glucose utilization and on the incorporation of radioactivity from pyruvate-[2-^{14}C] and citrate-[1,5-^{14}C] into trichothecin and trichothecolone at this stage in the fermentation. The incorporation of radioactivity from stearate-[U-^{14}C] was too low (0.03% into trichothecin and 0.003% into trichothecolone) for meaningful comment. The results are shown in Figs 2 and 3. There was a fall in the rate of utilization of glucose and a marked drop in the incorporation of radioactivity from citrate and pyruvate into both trichothecin and trichothecolone. In the case of citrate the effect was also shown to increase with mass. Since there was a drop in the rate of utilisation of glucose, these effects represent a repression of acetate biosynthesis rather than a drop in the incorporation arising by dilution of the acetyl CoA pool. Both pyruvate decarboxylase and citrate lyase are regulatory enzymes in their respective pathways.

Although multiple-labelling has been noted on several occasions in polyketide biosynthesis [8, 9], it is unusual in terpenoid biosynthesis [10]. To overcome these problems, the acetate-[1,2-$^{13}C_2$] was diluted with unlabelled material (1:2) and fed in nine three-hour pulses *T. roseum* from day 5 of the fermentation. The metabolites, which were isolated on day 7, had ^{13}C NMR spectra showing none of the secondary couplings which had been observed in the earlier experiment, whilst the MS (see Table 1, expt. 2) showed no evidence of multiple-labelling.

The coupling constants are given in Scheme 1 The coupling pattern, in particular the lack of coupling involving C-8 and the presence of coupling between C-6 and C-7 and between C-10 and C-11, is fully in accord

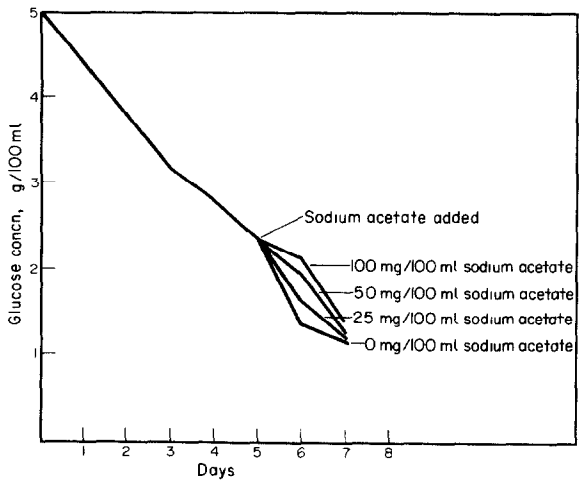
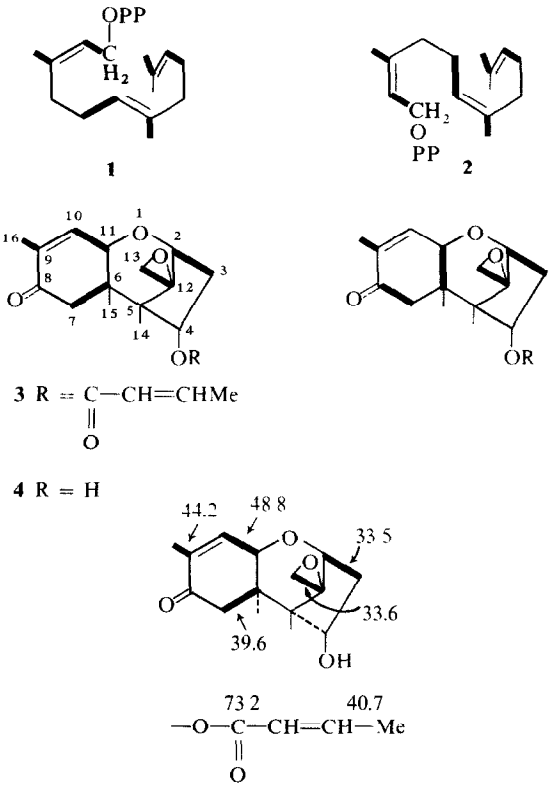


Fig. 2. Glucose utilization by *T. roseum*.

$^{13}CH_3$–$^{13}CO_2H$



Scheme 1. Coupling constants (Hz) for trichothecolone and trichothecin.

with the folding of farnesyl pyrophosphate (**1**) which we and others [11] had previously established. However it does require the revision of two pairs of assignments. Previously we had assigned the lower-field of the two >CH.O-doublets (C-2 and C-11; 70.2 and 79.6 ppm) to C-11 since it is allylic to the 9,10-double bond whilst the chemical shift of C-2 appeared to reflect variations in the substituents at C-3. However the coupling patterns show that these assignments must be reversed bringing them into accord with those of other workers [5–7]. Secondly, by analogy with the ^1H NMR spectrum, we had assigned the lowest field methyl quartet to the olefinic methyl (C-16). However the coupling pattern shows that the

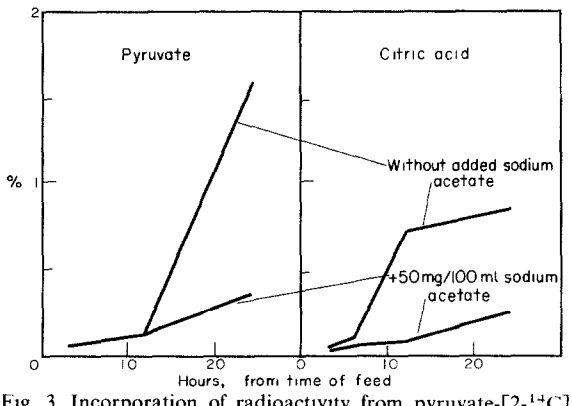


Fig. 3. Incorporation of radioactivity from pyruvate-[2-^{14}C] and citric acid-[1,5-^{14}C] into trichothecin.

Table 2. Revised ^{13}C NMR assignments of trichothecin and trichothecolone (in ppm from Me_4Si; $CDCl_3$ soln)

Carbon atom	Trichothecin*	Trichothecolone
2	79.6	79.2
3	36.95	40.1
4	73.3	73.0
5	49.0	49.1
6	43.7	43.3
7	42.1	42.1
8	198.6	198.8
9	138.1	138.0
10	137.1	137.3
11	70.1	69.8
12	65.5	65.6
13	47.4	47.2
14	5.7	5.9
15	18.5	18.3
16	15.4	15.4

* C-1′, 166.1; C-2′, 120.4; C-3′, 145.9; C-4′, 15.4.

methyl resonance at 15.4 ppm should be assigned to this carbon atom. The corrected ^{13}C NMR assignments are given in Table 2.

The appearance of trichothecolone (**4**) in the medium lags behind that of trichothecin (**3**) (see Fig. 1). However we had shown that trichothecolone was incorporated [12] into trichothecin in 27% yield. In the light of this, we have shown that trichothecin is hydrolysed by *T. roseum* to afford trichothecolone in 3.67% yield. It is also hydrolysed non-enzymatically by the medium (2.5%) over the 48 hr period. It is probable that the biosynthesis of trichothecin occurs within the mycelium and that the appearance of trichothecolone in the medium represents the sum of hydrolysis and the failure of the last enzymatic esterification step as the idiophase progresses.

EXPERIMENTAL

General methods Trichothecium roseum (CMI 50660) was grown on a medium comprising glucose (20 g, except where stated), ammonium tartrate (2 g), $MgSO_4.7H_2O$ (0.5 g), K_2HPO_4 (1.0 g), KCl (0.5 g), $FeSO_4.7H_2O$ (10 mg) and corn steep liquor (10 ml) per litre in conical flasks (100 ml per flask) at a shake rate of 125 rpm. The broth was filtered and extracted twice with half its vol. of $CHCl_3$. The extracts were dried and evapd *in vacuo* to afford an oil. The metabolites were separated by TLC on Si gel (Merck) in EtOAc–petrol (3:2) and crystallized from EtOAc–petrol (trichothecolone) or petrol (trichothecin). Quantitative measurements were made by GLC on 1% OV-17 at 260° or 5% SE-30 at 240° (1.5 m columns, N_2 carrier gas, 50 ml/min) against standard solns. ^{13}C NMR spectra were determined on a JEOL PFT 100 for solns in $CDCl_3$.

Optimization of trichothecene production. (a) Variation with time; 10 flasks of *T. roseum* were grown on shake culture. One flask was harvested each day and the yield (mg/l.) (see Fig. 1) of trichothecenes in the extract was determined by GLC. (b) Variation of acetate-[2-^{14}C] incorporation with age of culture: 10 flasks (100 ml) of *T. roseum* were grown on shake culture. On each day of growth, one culture was fed with Na acetate-[2-^{14}C] (10 mg, 8 μCi) in H_2O (0.1 ml). The cultures were allowed to grow for 2 days after feeding and were then harvested. The metabolites were separated by TLC and crystallized to constant radioactivity. (see Fig. 1). (c) Variation of the incorporation of acetate into trichothecin with glucose concn: media containing 12.5, 25 and 50 g/l. glucose were prepared and sterilized. Two shake flaks (100 ml) of each were inoculated with *T. roseum*. Na acetate-[2-^{14}C] (2 μCi) in H_2O (0.1 ml) was added to each flask after 5 days growth. The cultures were separately harvested after a further 2 days growth. The metabolites were isolated, separated by TLC and crystallized to constant radioactivity. The incorporations (%) observed for the increasing glucose concentrations were 0.07, 1.57 and 1.48 respectively in trichothecin and 0.03, 0.54 and 0.49 respectively in trichothecolone. (d) Variation of the incorporation of acetate into trichothecin with acetate concn: Na acetate-[2-^{14}C] (2 μCi) in H_2O (0.1 ml) together with unlabelled Na acetate (10, 50 or 100 mg) were added to 5 day old shake cultures of *T. roseum*. After a further 2 days growth, the cultures were harvested. The metabolites were isolated, separated and crystallized to constant radioactivity. The incorporations (%) found for the increasing acetate concs were 0.48, 0.43 and 0.23 respectively in trichothecin and 0.49, 0.44 and 0.36 respectively in trichothecolone.

The effect of exogenous acetate on T. roseum. (a) Na pyruvate-[2-^{14}C] (50 μCi) in H_2O (0.8 ml) was evenly distributed between 8 five-day old shake cultures (100 ml, 25 g/l. glucose) of *T. roseum*. Na acetate (50 mg) was also added to each flask. The flasks were harvested after 3, 6, 12 and 24 hr. The metabolites were isolated, separated and crystallized to constant radioactivity (see Fig. 3). (b) The above experiment was repeated with citric acid-[1,5-^{14}C] (50 μCi). The results are shown in Fig. 3. (c) citric acid-[1,5-^{14}C] (50 μCi) in H_2O (0.8 ml) was evenly distributed between 4 five-day old cultures (100 ml, 25 g/l. glucose) of *T. roseum*. Na acetate (25, 50 and 100 mg respectively) was added to each of 3 flasks. All 4 cultures were harvested after a further 2 days growth. The metabolites were isolated, separated and crystallized to constant radioactivity. (d) Thirteen shake flasks (100 ml, 50 g/l. glucose) were inoculated with *T. roseum*. After each day's growth one flask was filtered, the optical rotation of the broth was measured and thus the concn of the remaining glucose was determined. After 5 days growth Na acetate (2 × 25.2 × 50 and 2 × 100 mg respectively) was distributed between 6 flasks. One flask of each concn and one blank was analysed on each of the following 2 days. The results are shown in Fig. 2.

Incubation of Na acetate-[1,2-$^{13}C_2$] with T. roseum. (a) Na acetate-[1,2-$^{13}C_2$] (98% enriched at both centres) (200 mg) and Na acetate-[2-^{14}C] (4 μCi) in H_2O (1.2 ml) was distributed between 4 five-day old shake cultures (100 ml, 25 g/l. glucose) of *T. roseum*. After a further 2 days the cultures were harvested and the metabolites were purified to afford trichothecin (18.4 mg, 1.39% incorporation) and trichothecolone (16.0 mg, 0.9% incorporation). (b) Na acetate-[1,2-$^{13}C_2$] (200 mg), sodium acetate-[2-^{14}C] (23.4 μCi) and unlabelled Na acetate (400 mg) were dissolved in H_2O (3.6 ml). The soln was distributed in aliquots (0.1 ml) to each of 4 shake cultures of *T. roseum* (100 ml, 25 g/l. glucose) at *ca* 3 hr intervals from the fifth day of growth. The cultures were harvested after a total of 7 days growth. The metabolites were purified to afford trichothecin (17.9 mg, 0.16% incorporation) and trichothecolone (18.2 mg, 0.12% incorporation).

Recovery of unused Na acetate-[1,2-$^{13}C_2$]. An aliquot of the residual fermentation broth after the recovery of the metabolites, was counted. The broth was shown to contain *ca* 30% of the initial Na acetate. It was then concd under vacuum for a few min to remove any remaining organic solvent. The broth was acidified with conc HCl (0.1 ml per 100 ml broth) and applied to a column (1 × 20 cm) of Dowex 1-X8 ion exchange resin (chloride form) which had previously been washed with H_2O (200 ml). The efficient contained less than 5% of the applied radioactivity. The column was then washed with H_2O (100 ml). It was then eluted with ammonia (2M) and fractions (20 ml) were collected. These were counted and those containing radioactivity were pooled. The mass of acetate present was calculated and a slight excess of NaOH was added. The aq. soln was then evapd to dryness to yield the Na acetate (59.2 mg) δ ^{13}C 59.3 and 217.5, J = 51.9 Hz (in H_2O).

Hydrolysis of trichothecin. (a) ^{14}C Trichothecin (from mevalonate-[2-^{14}C]), (162300 dpm) in EtOH (0.5 ml) was added to a 2 day old shake culture (100 ml) of *T. roseum*. The culture was harvested after 7 days growth to afford a crude oil (77830 dpm,

47.9%). The metabolites were purified in the usual way to afford trichothecolone (5956 dpm, 3.67% incorporation). (b) Trichothecin (15 mg, 30096 dpm) in EtOH (0.5 ml) was added to the fermentation medium (100 ml). After 48 hr the products were recovered to afford unchanged trichothecin (28457 dpm, 94.5% recovery) and by dilution, trichothecolone (753 dpm, 2.5% incorporation).

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