Studies on Fungal Metabolites.[1] Part 2. Carbon-13 Nuclear Magnetic Resonance Biosynthetic Studies on Pentaketide Metabolites of *Aspergillus melleus*: 3-(1,2-Epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one and Mellein

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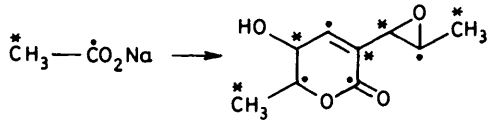
The ^{13}C n.m.r. spectra and incorporations of ^{13}C-labelled precursors into pyrone and dihydroisocoumarin pentaketide metabolites from the culture liquors of *Aspergillus melleus* are reported. Detection of a two-bond ^{13}C–^{13}C coupling in a metabolite enriched from [1,2-$^{13}C_2$]acetate provides proof for an intramolecular rearrangement occurring during biosynthesis.

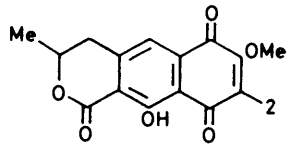
FERMENTATIONS of *Aspergillus melleus* provide a variety of metabolites: the mycelium is a rich source of polyketide-derived naphthoquinone pigments,[2,3] *e.g.* xanthomegnin (1), whereas the principle metabolite in the culture liquors is the unusual pyrone (2), a weak broad spectrum antibiotic which was first isolated [4] along with mellein (4) and penicillic acid (6), and has since been isolated from other *Aspergillus* species.[5] Incorporation of [^{14}C]acetate into pyrone (2) was reported [6] to give the labelling pattern shown in Scheme 1. The distribution of label, particularly the linkage of two carbons derived from the methyl carbon of acetate, and the branched structure of the pyrone are difficult to rationalise in terms of the normal pathways of polyketide biosynthesis. Studies based on ^{13}C-labelling have facilitated the elucidation of unusual biosynthetic pathways [7]



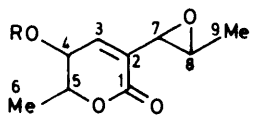
SCHEME 1 Incorporation pattern of acetate into *A. melleus* pyrone (2)

and ^{13}C n.m.r. studies of pyrone (2) and its co-metabolites are now reported.[8]

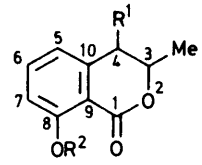
Before undertaking ^{13}C-enrichment studies it was necessary to have an unambiguous assignment of the ^{13}C n.m.r. spectrum, and a full assignment of the ^{13}C n.m.r. spectra of the pyrone (2) and its acetate (3) has been made as follows: C-1, C-2, C-3, and the 4-acetate carbons were readily assigned from standard chemical-shift data and their multiplicities in the single frequency off-resonance decoupled (s.f.o.r.d.) spectra. Carbons 4, 5, 7, and 8, however, all have similar chemical shifts, characteristic of oxygen-bearing aliphatic carbons, and give rise to doublets in the s.f.o.r.d. spectra. They were assigned for the acetate (3) by the Birdsall method,[9] in which the peak frequencies in s.f.o.r.d. spectra are plotted against the ^1H irradiating frequency as it is stepped through the ^1H spectral region. The corresponding ^1H and ^{13}C resonances are determined by extrapolating (Figure) the residual couplings to zero, and as the ^1H frequencies have been unambiguously assigned,[4] the corresponding ^{13}C resonances can be assigned. The 6- and 9-methyl resonances could not be readily distinguished in the natural abundance ^{13}C n.m.r. spectra. However in the spectrum of (2) enriched from [1,2-$^{13}C_2$]-acetate, the resonance at 17.6 p.p.m. shows a ^{13}C–^{13}C coupling of 44 Hz to the resonance at 59.1 p.p.m., which



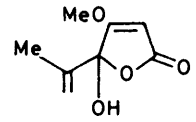
(1)



(2) R = H
(3) R = Ac



(4) $R^1 = R^2 = H$
(5) $R^1 = H$ $R^2 = Me$
(7) $R^1 = OH$ $R^2 = H$



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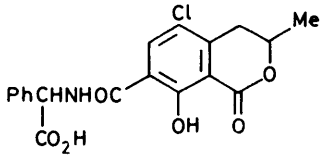

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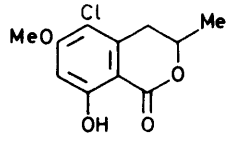

(9)

has been unambiguously assigned to C-8, and so the former must be assigned to C-9.

The ^{13}C n.m.r. spectra of (2) enriched from feedings of [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetate, and [2-^{13}C]malonate are summarised in Table 1. High enrichment of carbons 1, 3, 5, and 8 from [1-^{13}C]acetate, and carbons 2, 4, 6, 7, and 9 from [2-^{13}C]acetate were appa-

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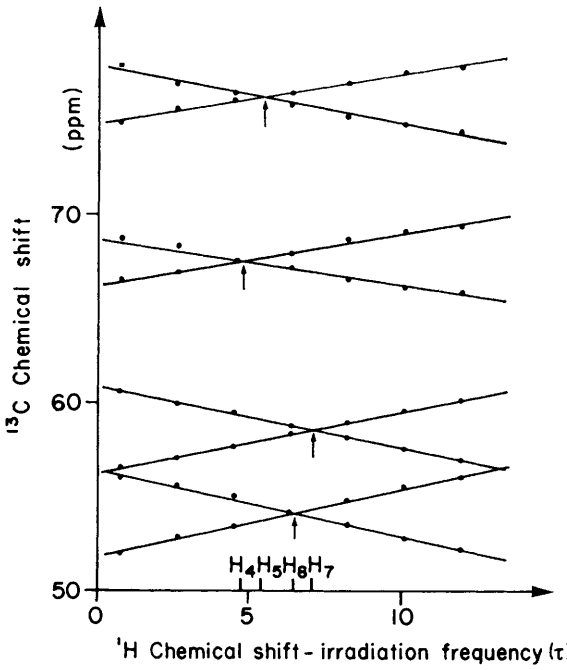
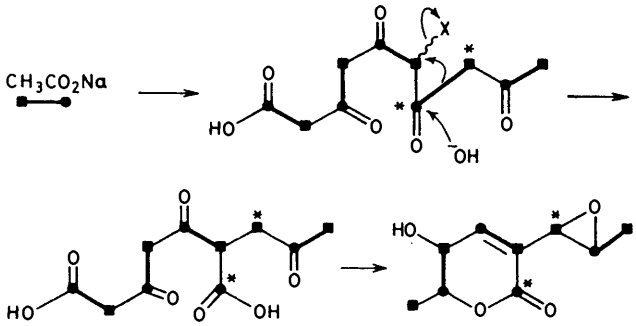
rent, confirming the previously reported labelling pattern. The specific incorporation of [2-^{13}C]acetate was sufficiently high for satellites due to a ^{13}C–^{13}C coupling of 61 Hz, corresponding to the head-to-head linkage of acetate units, to be observed on the resonances due to carbons 2 and 7. It should be noted that the overall



Plot of peak frequencies in the ^{1}H off-resonance selectively decoupled ^{13}C spectra of (3) as a function of positionof irradiation in the ^{1}H spectrum

enrichment, *ca.* 4.5 atom % ^{13}C, is much too low to allow such satellites to be observed so a significant proportion of the enriched molecules must be multiply labelled, with the remaining molecules having a much lower level of enrichment to give the observed overall level of enrichment.[10]

The ^{13}C n.m.r. spectrum of [1,2-$^{13}C_2$]acetate-enriched pyrone (2) shows ^{13}C–^{13}C couplings between the following pairs of carbons: 2 and 3 (68 Hz), 4 and 5 (41 Hz), and 8 and 9 (44 Hz) thus proving their origin from acetate units which have remained intact throughout the biosynthetic sequence to give the labelling pattern shown in Scheme 2. On redetermining the spectrum using 500 Hz sweep widths, an additional small coupling of 6 Hz was resolved on the resonances due to carbons 1 and 7. Thus carbons 1 and 7 must also be derived from an originally intact acetate molecule which has undergone an *intra*molecular rearrangement during the course of



SCHEME 2 Incorporation of [1,2-$^{13}C_2$]acetate into the pyrone (2) *via* intramolecular rearrangement of a pentaketide precursor

biosynthesis. The above enrichment data is consistent with the biosynthetic pathway shown in Scheme 2, and indicate that pyrone (2) is formed from a pentaketide precursor *via* a Favorskii-type rearrangement; generating both the observed head-to-head linkage of acetate units, and the 1,3-coupling between carbons 1 and 7; followed by loss of the terminal carboxy-group, a biosynthetically unexceptional step. A similar rearrangement can be postulated to account for the formation of the fused bis-furan ring system found in the aflatoxins and related metabolites, whose origin has been a subject of much research and speculation.[11] Since our preliminary report,[8] a similar rearrangement has been shown to occur in the biosynthesis of vulgamycin,[12] and a furanoid metabolite of *Chaetomium coarcatum*.[13]

TABLE 1

^{13}C Chemical shifts (δ_C, p.p.m. downfield from internal $SiMe_4$) in pyrones (2) and (3), and enrichments observed in ^{13}C incorporation experiments

Carbon	(2) [a]	Observed enrichments from [1-^{13}C]-acetate [b]	[2-^{13}C]-acetate [c]	[2-^{13}C]-malonate [d]	^{13}C–^{13}C couplings (J Hz) from [1,2-$^{13}C_2$]acetate	(3) [a]
1	163.0 (s)	+			6	161.7 (s)
2	129.0 (s)		+ [e]	+	68	131.2 (s)
3	141.2 (d)	+			68	135.5 (d)
4	67.6 (d)		+	+	41	67.8 (d)
5	79.3 (d)	+			41	76.3 (d)
6	18.0 (q)		+	+	—	18.1 (q)
7	54.6 (d)		+ [e]	+	6	54.3 (d)
8	59.1 (d)	+			44	58.7 (d)
9	17.6 (q)		+	+	44	17.5 (q)
CH_3CO	—					20.6 (q)
CH_3CO	—					169.4 (s)

[a] Multiplicities refer to off-resonance decoupled spectra. [b,c,d] Average enrichments are + 13, 4.5, and 0.3 atom % ^{13}C respectively. [e] ^1J(^{13}C–^{13}C) 61 Hz observed.

Incorporation of [2-^{13}C]malonate resulted in very low overall enrichment and no ' starter ' effect was observed, C-9 being enriched to the same level as the other carbons derived from the methyl carbon of acetate.

In addition to the pyrone (2), variable yields of mellein (4) and penicillic acid (6), the previously reported co-metabolites,[4] were obtained from the *A. melleus* fermentations. However, a further metabolite was isolated from the fermentation liquors. From its spectral and physical properties it was identified as *cis*-4-hydroxymellein (7) which has been isolated from several fungi, including *Lasiodiplodia theobromae*,[14] and *Cercospora taiwanensis*.[15]

The ^{13}C n.m.r. spectra of mellein (4), *O*-methylmellein (5), and (7) are summarised in Table 2.

TABLE 2

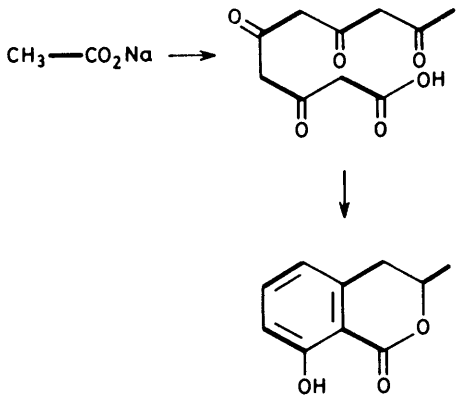
^{13}C Chemical shifts (δ_C, p.p.m. downfield from internal $SiMe_4$) and coupling constants of [1,2-^{13}C]acetate-enriched (4) and ^{13}C chemical shifts of compounds (5) and (7).

	(4)		(5)	(7)
Carbon	δ_C	J/Hz	δ_C	δ_C
1	169.7 (s)	68	162.6 (s)	168.4 (s)
3	76.0 (d)	40	74.0 (d)	80.0 (d)
4	34.6 (t)	41	36.1 (t)	69.0 (d)
5	117.8 (d)	55	119.1 (d)	117.6 (d)
6	135.9 (d)	55	134.4 (d)	136.7 (d)
7	116.1 (d)	67	110.9 (d)	116.2 (d)
8	162.1 (s)	67	161.0 (s)	161.7 (s)
9	108.2 (s)	68	113.6 (s)	106.5 (s)
10	139.2 (s)	41	141.8 (s)	141.0 (s)
11	20.7 (q)	40	20.6 (q)	17.9 (q)
MeO			56.1 (q)	

Multiplicities refer to off-resonance decoupled spectra.

The ^{13}C resonances were assigned from standard shift values, multiplicities in s.f.o.r.d. spectra, and by analysis of the long-range ^{1}H–^{13}C couplings in fully ^{1}H-coupled ^{13}C n.m.r. spectra. This is particularly useful for distinguishing the 5 and 7 carbons which are of similar chemical shift in all three compounds. C-6, as expected, appears as a simple doublet due to one-bond coupling to H-6, typically 162 Hz in (7). In all three compounds C-7 appears as a doublet of doublets due to one bond coupling to H-7 and a three-bond coupling to H-5, typically 166 and 7 Hz respectively in (7). However in both (4) and (5) C-5 appears as a doublet of quintets due to the large one-bond coupling to H-5, with the 5-line pattern arising from a 3-bond coupling of *ca.* 7 Hz to H-7 and a *ca.* 3.5 Hz coupling to the 4-methylene protons; selective low-power irradiation of the 4-protons collapses the quintets to doublets. Similarly C-10 shows a 3-bond coupling of *ca.* 7 Hz to H-6 with 2- and 3-bond couplings of *ca.* 3.5 Hz to the 4- and 3-protons respectively so that, for example, irradiation of the 4-methylene protons in (5) causes the broad poorly resolved multiplet due to C-10 to sharpen to a doublet of doublets (J 7 and 3.5 Hz) and irradiation of H-4 causes it to sharpen to a doublet of triplets. In compound (7) on the other hand C-5 appears as a doublet of triplets due to the one-bond coupling (162 Hz) to H-5 and equal three-bond couplings to H-7 and H-4 (7 Hz); C-10 appears as a doublet of poorly resolved triplets due to a 7 Hz coupling to H-6 and smaller couplings to H-4 and H-3.

On methylation of (4) to give (5), the removal of chelation between the *peri*-related hydroxy and carbonyl groups results in characteristic changes in chemical shift.[16] Sufficient mellein was isolated from the [1,2-$^{13}C_2$]-acetate fermentation for its doubly labelled ^{13}C n.m.r. spectrum to be determined, and the observed ^{13}C–^{13}C couplings (Table 2) confirm that mellein is formed by the anticipated folding of a pentaketide chain (Scheme 3). A



SCHEME 3 Incorporation of [1,2-$^{13}C_2$]acetate into mellein

similar acetate assembly pattern has been found for the related dihydroisocoumarin (9), a metabolite of *Sporamia affinis*.[17]

It is noteworthy that both mellein (4) and the pyrone (2) lack the equivalent oxygen atom from the precursor pentaketide chain. Moreover, on repeated culture of *A. melleus* yields of the pyrone decreased with concomitant increase yields of mellein which was originally produced in very low yields. This suggests that both compounds are derived from a common deoxypentaketide which is diverted to mellein production when pyrone production is inhibited. Pyrone (2) has also been isolated from *A. ochraceus* [5c] which also produces the ochratoxins [*e.g.* (8)], dihydroisocoumarins whose biosynthesis must also involve a deoxypentaketide precursor.

EXPERIMENTAL

For general experimental details see part 1.

^{13}C *N.m.r. Determinations.*—The ^{13}C n.m.r. spectra were obtained for samples in acid-free $CDCl_3$ with $SiMe_4$ as internal reference. Proton noise-decoupled and single frequency off-resonance decoupled spectra were determined on a Varian XL100-15FT spectrometer operating at 25.197 MHz or on a JOEL JNMFX-60 spectrometer operating at 15.04 MHz. Fully coupled spectra were determined under gated-1 decoupling conditions to retain nuclear Overhauser effects.

Isolation of Metabolites.—Aspergillus melleus (CMI 49108) was grown at 25 °C in static culture in penicillin flasks each containing 500 ml of an aqueous medium made up from potassium dihydrogen phosphate (0.1%), magnesium sulphate heptahydrate (0.05%), potassium chloride

(0.05%), urea (0.07%), and glucose (7.5%), the solution being adjusted to pH 5 before autoclaving. The fermentation was harvested after 18 days growth and the culture filtrate was extracted with ethyl acetate to give a brown gum (*ca.* 400 mg l^{-1}) which was purified by preparative t.l.c. on 20 × 20 cm silica-gel plates eluted with ether–light petroleum (60 : 40, v/v). The three u.v.-quenching bands were removed to give in order of decreasing polarity (a) 3-(1,2-epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one, m.p. 109—111 °C (lit.,[4] m.p. 109—111 °C) (*ca.* 240 mg l^{-1}); (b) 4-hydroxymellein (*ca.* 20 mg l^{-1}), m.p. 114—117 °C (lit.,[14] m.p. 112—117 °C); and (c) mellein (*ca.* 10 mg l^{-1}), m.p. 54—56 °C (lit.,[18] m.p. 55—56 °C). In later fermentations yields of the pyrone decreased with concomitant increase in the yields of mellein.

Incorporations of ^{13}C *Labelled Precursors.*—To each of three vessels containing a 7-day growth of *A. melleus* was added 90% sodium [1-^{13}C]acetate (400 mg), sodium [2-^{13}C]-acetate (250 mg), and sodium [1,2-$^{13}C_2$]acetate (250 mg). After a further 4 days growth the liquors were extracted to give pyrone (2), in yields of 25, 39, and 37 mg from the [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetate feeds respectively. In addition, mellein (15 mg) was isolated from the [1,2-$^{13}C_2$]acetate feed.

Diethyl [2-^{13}C]malonate (250 mg) in ethanol (1 ml) was similarly added to a 7-day old culture of *A. melleus* and pyrone (2) (30 mg) was isolated after a further 4 days growth.

Acetylation of the Pyrone (2).—The pyrone (100 mg) in acetic anhydride (2 ml) and pyridine (0.5 ml) was stirred at room temperature for 2 h. Work-up gave an almost quantitative yield of the acetate (3), m.p. 65—67 °C (lit.,[4] m.p. 65—67 °C).

Methylation of Mellein.—Mellein (100 mg) was stirred at room temperature overnight in the presence of chloroform (10 ml), methyl iodide (1 ml), and silver oxide (500 mg). Filtration of solids and removal of solvent gave an oil which crystallised from ethyl acetate to give *O*-methylmellein, m.p. 88—89 °C (lit.,[18] m.p. 88—89 °C).

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