A ^{2}H and ^{13}C N.M.R. Study of the Biosynthesis of the Polyketide Isocoumarin Residue of Canescin in *Aspergillus malignus* from [1,2-$^{13}C_2$]-, and [1-^{13}C, 2-$^{2}H_3$]Acetates, [*Me*-^{13}C, $^{2}H_3$]Methionine, 6,8-Dihydroxy-3,7-dimethylisocoumarin, and 6,8-Dihydroxy-7-formyl-3-methylisocoumarin

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Evidence is presented that 6,8-dihydroxy-3,7-dimethylisocoumarin (**8**) and 6,8-dihydroxy-7-formyl-3-methylisocoumarin (**9**) are advanced precursors of the polyketide residue of canescin in *Aspergillus malignus*.

Canescin (**1**), a metabolite of *Aspergillus malignus* and *Penicillium canescens*, has been shown by Birch to have a mixed biosynthetic origin.[1] The isocoumarin portion of the molecule is derived from acetate *via* a polyketide chain; the carbon atoms of the methoxy group and C-10 are both derived from methionine; the remaining three carbons C-11, C-12, and C-13 probably derive from one of the C_4 intermediates of the citric acid cycle. We now report experiments using stable isotopes which throw light on the early steps of the biosynthetic pathway.

Canescin is isolated as a mixture of diastereoisomers, which give rise to a complicated ^{13}C n.m.r. spectrum in which many

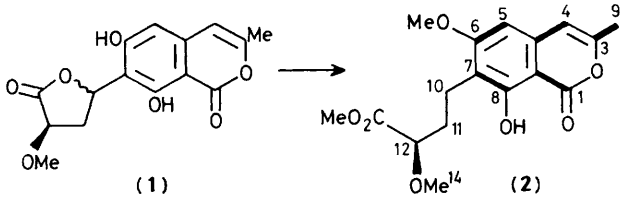
of the peaks appear doubled. Therefore the natural product was treated with sodium borohydride, followed by methylation of the resulting acid with diazomethane to give the derivative (**2**) in which one of the chiral centres has been destroyed (Scheme 1). The ^{13}C n.m.r. spectrum of this derivative was assigned by standard chemical shift arguments and single frequency decoupling experiments (Table 1).

Sodium [1,2-$^{13}C_2$]acetate and [*Me*-^{13}C,$^2H_3$]methionine were administered to the growing organism in separate experiments. In the ^{13}C n.m.r. spectrum of the derivative (**2**) produced in the first experiment each carbon atom of the isocoumarin nucleus gave rise to a ^{13}C–^{13}C doublet in addition to the natural abundance singlet; the doublets could be paired unambiguously by coupling constant (Table 1) and the resulting labelling pattern is consistent with a pentaketide origin for this part of the molecule as indicated on (**2**).

The material from the methionine feeding gave a ^{13}C n.m.r. spectrum (^1H,^2H decoupled) in which α-shifted peaks[2] were visible for the methoxy group and for C-10. The magnitude of the shifts, and the multiplicities of the signals in a proton-coupled spectrum, showed that at these sites three and one deuterium atoms, respectively, had been retained. The retention of deuterium at C-10 rules out any biosynthetic pathway in which this residue has been oxidised to the carboxylic acid level, as is the case in ochratoxin biosynthesis.[3]

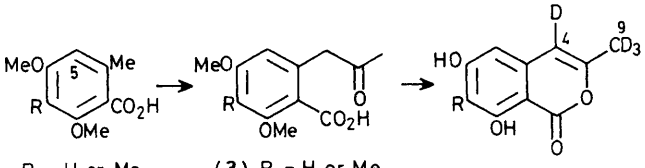
Next the isocoumarin precursors were prepared as indicated in Scheme 2. The ketoacids represented by (**3**) were synthesised from the corresponding 6-methylsalicylic acids *via* a carboxylation, acylation, and decarboxylation sequence.[4] They were then treated with NaOD in D_2O, followed by acid-catalysed ring closure,[5] and deprotection to give (**4**) and (**5**). The partially deprotected isocoumarin (**6**) was formed analogously to (**4**), but with only partial demethylation using Me_3SiI with D_2O work-up (^2H was incorporated at an additional site, 5-H, under the acid conditions of the work-up). This was converted into the labelled aldehyde (**7**) by formylation,[6] followed by demethylation (BBr_3). The distribution of deuterium in the isocoumarins was determined from the ^1H n.m.r. spectra (Table 2). In each case a satisfactorily high proportion of the sample was intramolecularly labelled with ^2H at two or more sites, as was required to test for intact incorporation in the biosynthetic experiments.

The canescin derivative (**2**) produced after incorporation of (**5**) showed in its mass spectrum a peak at *m/z* 340 ($M^+ + 4$) about 15% of the size of that at *m/z* 336 (M^+), whilst that at *m/z* 339 was *ca.* 2%. Taking into account the amount of the precursor consumed (74 mg), and the amount of derivative (**2**) isolated (68 mg), we calculate that at least 6% of the labelled precursor was incorporated intact into the metabolite (**1**), a level which strongly supports the status of (**8**) as a true biosynthetic intermediate. The location of the ^2H labels was confirmed by the ^2H n.m.r. spectrum which showed two

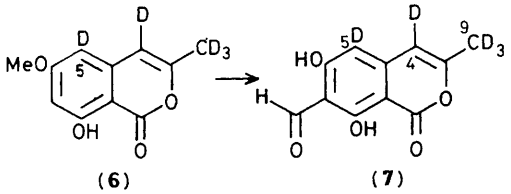


(**1**) (**2**)

Scheme 1



R = H or Me (**3**) R = H or Me (**4**) R = H (**5**) R = Me



(**6**) (**7**)

Scheme 2

Table 1. ^{13}C N.m.r. data[a] for canescin derivative (**2**).

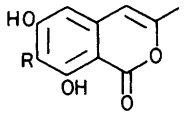
Carbon atom	δ	$J(^{13}C–^{13}C)$[b]/Hz	α-shift[c]/p.p.m.
13	173.1	61.8[d]	
1	166.5	73.4	
6	164.6	67.6	
8	160.3	72.3	
3	153.8	53.5	
4a	137.6	54.6	
7	115.6	72.2	
4	104.7	54.4	
8a	100.0	73.4	
5	97.0	67.9	
12	80.6	61.9[d]	
14	58.0		0.9
ArOMe	55.6		
CO_2Me	51.6		
11	31.5		
9	19.3	53.5	
10	18.7		0.3

[a] Relative to Me_4Si in a $CDCl_3$ solution. [b] In (**2**) derived from sodium [1,2-$^{13}C_2$]acetate. [c] In (**2**) derived from [^{13}C,$^2H_3$]methionine. [d] These satellites were of lower relative intensity than the others.

Table 2. Incorporation experiments with ^2H labelled isocoumarin precursors.

			Amounts of ^2H at indicated sites[a]		
			Precursor[c]		Derivative (**2**)[d]
Expt.	Precursor	Incorporation level/%[b]	4-H	5-H	4-H + 5-H
1	(**5**)	6	0.3	0	0.26
2	(**6**)	1	0.16	0.13	0.18
3	(**4**)	<0.1	0.28	0	—
4	$CD_3{}^{13}CO_2H$	0.6	—	—	—

[a] Relative to 9-H position (=1); in the isocoumarin precursors deuteriation of the 9-H position was essentially complete. [b] Based on mass spectrometry. [c] Based on ^1H n.m.r. data. [d] Based on ^2H n.m.r. data.



(**8**) R = Me
(**9**) R = CHO

singlets corresponding to the methyl (9-H) and vinyl (4-H) positions (the latter overlapped with the signal from the 5-H position in the ^{2}H n.m.r. spectrum); the isotopic ratio for the two sites, as measured by the relative intensities of the two resonances, was little changed (experiment 1, Table 2).

For comparison, a control experiment (expt. 4) with $CD_3{}^{13}CO_2H$ as precursor was carried out: 9-H of the resultant (**2**) was labelled (^{2}H n.m.r. spectrum and the β-shift technique[7]), but not the other methyl-derived sites 4-H, and 5-H, presumably because those sites suffered loss of deuterium by exchange with the medium in early intermediates of the pathway. In view of this observation, we suggest that the change in the isotopic ratio in favour of the 9-H position in the first experiment may be attributed to a small degree of competing indirect incorporation of ^{2}H, resulting from breakdown of the precursor to produce ^{2}H labelled acetate. A similar effect was observed in a study of citrinin biosynthesis.[8]

The results for the aldehyde analogue (**7**) (expt. 2) proved that this isocoumarin can also be incorporated intact. Thus there were peaks for $M^+ + 4$ and $M^+ + 5$ ions in the mass spectrum of the derived (**2**), whose relative intensities (2.2 : 1) were the same as those of the precursor. From their intensity relative to the parent ion we calculate that at least 1% of the precursor was incorporated intact. The ^{2}H n.m.r. spectrum confirmed that the labels were retained at the expected sites, although in this case there was evidence from the more pronounced change in relative intensities (9-H : 4-H + 5-H) that competing indirect incorporation *via* degradation to acetate had taken place to a greater extent than in expt. 1.

In contrast, the derivative (**2**) isolated from the experiment with (**4**) gave no evidence for intact incorporation in either the mass spectrum or the ^{2}H n.m.r. spectrum (expt. 3), at the limits of detection of those techniques.

On the basis of these results we propose that the methylated isocoumarin (**8**) is the first enzyme free intermediate in the biosynthesis of the polyketide isocoumarin residue of canescin. Subsequent steps must involve oxidation of the C-10 methyl group to the aldehyde level to give (**9**), then probably condensation with a suitable C_3 or C_4 fragment which provides the rest of the γ-lactone residue.

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