Biosynthesis of Spicifernin, a Unique Metabolite of the Phytopathogenic Fungus, *Cochliobolus spicifer*

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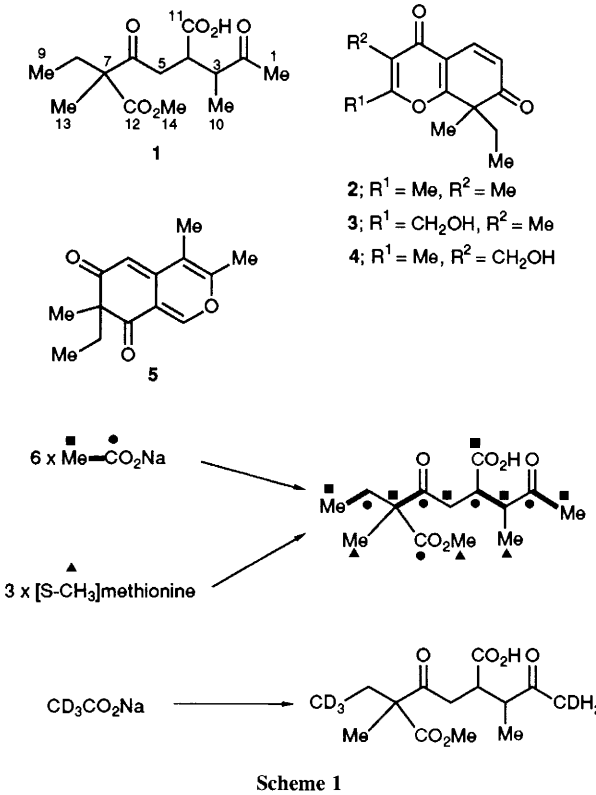
The biosynthesis of spicifernin **1** has been studied by feeding ^{13}C- and ^{2}H-labelled precursors to *Cochliobolus spicifer* cultures; the labelling pattern demonstrated by ^{13}C NMR spectroscopic analysis of the enriched products indicates that spicifernin is derived from a single polyketide chain bearing two *C*-methyl groups from C_1 units which undergoes double oxidative C–C bond cleavage and a decarboxylation.

A strain of *Cochliobolus spicifer* Nelson (D-5), a pathogen of leaf spot disease in wheat, produces several phytotoxins and a plant growth promoter simultaneously. The phytotoxins, *i.e.* spiciferones A **2**, B **3** and C **4**, and spiciferinone **5**, and the plant growth promoter, *i.e.* spicifernin **1**, have been isolated and characterized.[1] In spite of their different carbon skeletons, they have the following unique structural features in common: (*i*) a quaternary carbon bearing an ethyl, a methyl and a ketonic carbonyl, and (*ii*) vicinal methyls. This suggests strongly that these unique metabolites have the same origin.

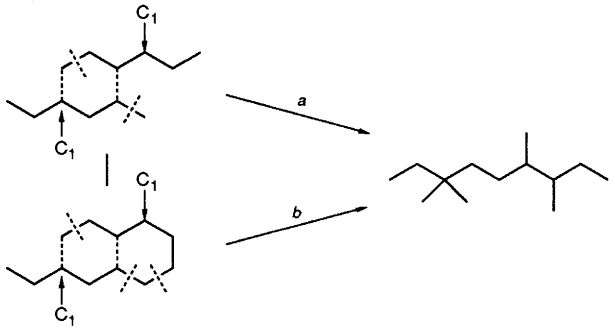
Table 1 ^{13}C NMR data[a] for methyl spicifernin

Carbon	δ_C	J_{CC}/Hz[b]	Relative enrichment [1-^{13}C]Acetate	[2-^{13}C]Acetate	[$^{13}CH_3$]Methionine	Isotopic[c] shift/ppm
1	28.4	41.2	1.39	3.41	1.29	
2	209.8	41.2	2.55	0.97	1.17	−0.11
3	46.5	34.3	1.18	2.73	0.88	
4	40.9	34.3	3.01	0.79	1.00	
5	35.8		1.13	2.42	1.22	
6	206.1	38.1	2.66	0.98	1.07	
7	59.7	38.1	0.97	2.42	0.96	
8	27.9	35.1	2.97	1.15	1.28	−0.08, −0.16, −0.23
9	8.4	35.1	1.31	2.83	1.14	
10	12.8		1.27	1.04	12.41	
11	173.8		1.08	1.96	1.00	
12	173.2		2.97	1.06	1.06	
13	18.3		1.33	1.11	13.18	
11-OMe	52.0		1.00	1.00	1.00	
12-OMe	52.3		1.49	1.30	10.70	

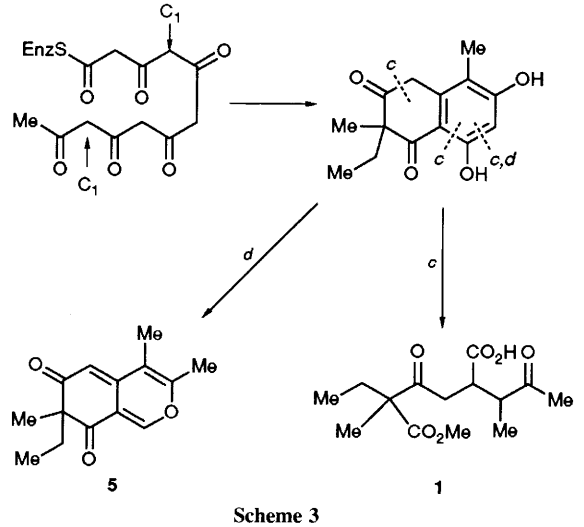
[a] At 100.5 MHz in $CDCl_3$. [b] After incorporation of [1,2-$^{13}C_2$]acetate. [c] After incorporation of [1-^{13}C,$^2H_3$]acetate.



Scheme 1



Scheme 2



Scheme 3

In order to explore this possibility, we started to study the biogenetic origins of spicifernin, a major metabolite of this fungus. Generally, production of phytotoxins by pathogens is involved in the infection process into their hosts,[2] and this fungus provides a good model for studying the regulation of secondary metabolism related to their pathogenesis.

^{13}C-Labelled precursors were supplied to 6 day old surface cultures of *C. spicifer* grown on a medium (100 ml × 15) containing glucose (30 g dm^{-3}), peptone (3 g dm^{-3}) and the extract from 100 g dm^{-3} of malt, and water at 24 °C every 24 h from day 6 to day 10. After a further 10 days, the cultures were filtered and solvent fractionation of the filtrate with EtOAc gave EtOAc-soluble acidic fraction. Silica gel and Sephadex LH-20 column chromatography of the fraction and subsequent purification by HPLC gave spicifernin **1** in yields of 10–20 mg per dm^3 of medium. The ^{13}C NMR spectra were measured in $CDCl_3$ after methylation with diazomethane.

The results of the feeding experiments are summarized in Table 1 and Scheme 1. Incorporation of [1,2-$^{13}C_2$]acetate indicated that the eight carbons were derived from intact acetate units. The results of incorporating [1-^{13}C]- and [2-^{13}C]-acetates are particularly interesting. As expected, C-2, C-4, C-6 and C-8 were enriched by [1-^{13}C]acetate, and C-1, C-3, C-7 and C-9 were enriched by [2-^{13}C]acetate. However, C-5 and C-11 were enriched by [2-^{13}C]acetate and C-12 was enriched by [1-^{13}C]acetate. Incorporation of [*S*-$^{13}CH_3$]-methionine indicated that C-10, C-13 and C-14 were derived from C_1 units. Results from our labelling studies with ^{13}C-labelled precursors suggested two possible routes to spicifernin; (*a*) a route from two triketide chains and (*b*) a

route from a single hexaketide chain, as shown in Scheme 2. If a two-chain pathway is operative, then both C-1 and C-9 are derived from the methyl carbon of an acetate 'starter' unit, whereas if a single-chain route operates then only C-9 is derived from a 'starter' acetate, C-1 being formed from a chain-propagation malonate molecule, following cleavage of a pre-formed cyclic intermediate. Thus by feeding of [1-^{13}C,$^{2}H_3$]acetate, it should be possible to distinguish between these two pathways. In the ^{13}C NMR spectrum of methyl spicifernin enriched with [1-^{13}C,$^{2}H_3$]acetate, deuterium-induced β-isotope shifts[3] were detected with two resonances due to C-2 and C-8. C-2 shows one isotopically shifted resonance (△ −0.11 ppm) corresponding to the incorporation of one deuterium atom at C-1, and C-8 shows three isotopically shifted resonances (△ −0.08, −0.16 and −0.23 ppm) corresponding to the incorporation of one, two and (mainly) three deuterium atoms at C-9, respectively, indicating its origin from an acetate 'starter' unit. Thus, a pathway from two triketide chains (route *a*) was excluded.

To account for these results, we propose the pathway shown in Scheme 3. A single hexaketide chain bearing two *C*-methyl groups from C_1 units is folded to give a bicyclic aromatic intermediate, which is then subject to double oxidative C–C bond cleavage, decarboxylation (path *c*) and further introduction of a C_1 unit into the carboxyl function to produce spicifernin. If the bicyclic intermediate is modified by an oxidative C–C bond cleavage and subsequent cyclization (path *d*), then spiciferinone will form. Several fungal metabolites were reported to arise by cleavage of aromatic rings.[4] However, such complex modification of a polyketide chain *via* an aromatic intermediate as in path *c* is very rare, but can be seen in the biosynthesis of terrein in which double decarboxylation occurs with an aromatic precursor. Ceratenolone[5] from *Ceratocystis minor* and similin B[6] from *Sporormiella similis* are structurally similar fungal metabolites to spiciferinone and perhaps they may arise from the same aromatic intermediate as in the biosynthesis of spicifernin and spiciferinone. The biogenetic relationship between spicifernin and spiciferones is also an interesting issue from the phytopathological point of view, and biosynthetic studies on spiciferones are currently in progress.

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