Biosynthesis of Spiciferone A and Spicifernin, Bioactive Metabolites of the Phytopathogenic Fungus, *Cochliobolus spicifer*

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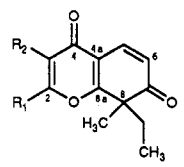
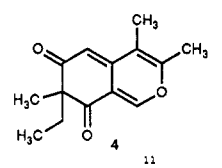
Summary: The biosynthetic pathways to spiciferone A and spicifernin were investigated by means of incorporation experiments with [1-^{13}C], [2-^{13}C], [1,2-^{13}C], and [1-^{13}C,^{2}H$_3$]acetate and [*S*-^{13}CH$_3$]-L-methionine. The incorporation patterns suggested that they are produced from a common precursor, which is derived from a hexaketide and two C_1 units, after undergoing modifications including the unique C–C bond cleavage by retro-aldol condensation.

The 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 (**1**), B (**2**), and C (**3**) and spiciferinone (**4**), and the plant growth promoter, *i.e.*, spicifernin (**5**), have been isolated and characterized.[1-4]



1 R_1=CH_3, R_2=CH_3
2 R_1=CH_2OH, R_2=CH_3
3 R_1=CH_3, R_2=CH_2OH



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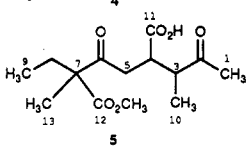

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Despite different carbon skeletons, they have the following unique structural features in common: (*i*) a quaternary carbon bearing an ethyl, a methyl, and a ketonic carbonyl and (*ii*) vicinal methyls. This commonality strongly suggests that these unique metabolites have the same origin. To explore this possibility, we undertook studies to investigate the biogenetic origins of spiciferone A and spicifernin, major metabolites of this fungus, and quite recently we reported preliminary results of incorporation experiments with spicifernin.[5] However, poor yields of spiciferone A on administration of labeled acetates have hampered our attempts to clarify the biogenetic origin of spiciferone A. During the continuing feeding experiments, we found that addition of methionine (300 mg/L) increased the yield of spiciferone A up to four times more than without methionine. We have thus used this procedure to overcome the previous difficulties and obtain full data concerning the origin of spiciferone A.

In this paper we report the incorporation patterns of labeled precursors into spiciferone A and spicifernin,[6] which indicate their common biogenetic origin, and we propose the biosynthetic pathways to spiciferone A and spicifernin based on the labeling patterns.

The results of the feeding experiments with spiciferone A are summarized in Table I and Scheme I. Incorporation of [1,2-^{13}C]acetate indicated that 12 carbons were derived from intact acetate units. The six carbons (C-2, C-4, C-5, C-7, C-8a, and the methylene carbon of Et-8) were enriched by [1-^{13}C]acetate, and the other six carbons (C-3, C-4a, C-6, C-8, Me-2, and the methyl carbon of Et-8) were enriched by [2-^{13}C]acetate. Incorporation of [*S*-^{13}CH$_3$]-L-methionine indicated that the remaining two carbons (Me-3 and Me-8) were derived from C_1 units. The results of the feeding experiments with specifernin have already been reported[5] and summarized in Scheme I.

Results from our labeling studies with ^{13}C-labeled precursors indicated that spiciferone A and spicifernin arise from the same origin and also suggested two possible routes to these metabolites: (a) a route from two triketide chains and (b) a route from a single hexaketide chain, as shown in Scheme II. If a two-chain pathway is operative, then both Me-2 and the methyl of Et-8 in spiciferone A, and also both C-1 and C-9 in spicifernin, are derived from the methyl carbon of an acetate "starter" unit, whereas if a single-chain route operates then only the methyl of Et-8 in spiciferone A and only C-9 in spicifernin are derived from the methyl carbon of a "starter" acetate. 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 spiciferone A enriched with [1-^{13}C,^{2}H$_3$]acetate, deuterium-induced β-isotope shifts were detected with resonance attributed to the methylene carbon of Et-8, which shows three isotopically shifted resonances (Δ-0.09, –0.17, –0.26 ppm) corresponding to the incorporation of one, two, and (mainly) three deuterium atoms on the methyl carbon of Et-8. The result of the feeding experiment with spicifernin indicated the incorporation of one deuterium atom at C-1 and three deuterium atoms at C-9.[5] These data indicate their origin from only one 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 III. A single hexaketide chain bearing two *C*-methyls from C_1 units is folded to give a 10-membered monocyclic intermediate **6**. If this intermediate

(1) Nakajima, H.; Hamasaki, T.; Kimura, Y. *Agric. Biol. Chem.* **1989**, *53*, 2297–2299.
(2) Nakajima, H.; Hamasaki, T.; Maeta, S.; Kimura, Y.; Takeuchi, Y. *Phytochemistry* **1990**, *29*, 1739–1743.
(3) Nakajima, H.; Hamasaki, T.; Kohno, M.; Kimura, Y. *Phytochemistry* **1991**, *30*, 2563–2565.
(4) Nakajima, H.; Kimura, Y.; Hamasaki, T. *Phytochemistry* **1992**, *31*, 105–107.
(5) Nakajima, H.; Matsumoto, R.; Kimura, Y.; Hamasaki, T. *J. Chem. Soc., Chem. Commun.* **1992**, 1654–1656.

(6) *C. spicifer* was grown on a medium (100 mL × 15) containing glucose (30 g/L), peptone (3 g/L), the extract from 100 g/L of malt, with or without L-methionine (300 g/L), and water at 24 °C without shaking. ^{13}C-Labeled precursors were supplied to 6-day-old cultures 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 neutral and acidic fractions. Silica gel column chromatography (10% acetone in *n*-hexane) of the neutral fraction and subsequent purification by HPLC (ODS, 70% aqueous MeOH) gave spiciferone A (**1**) in yields of 4–7 mg/L of medium. Silica gel partition column chromatography (10 and 20% EtOAc in *n*-hexane, saturated with 0.1 M HCOOH) and Sephadex LH-20 column chromatography (MeOH) of the acidic fraction and subsequent purification by HPLC (ODS, 70% aqueous MeOH containing 1% AcOH) gave spicifernin (**5**) in yields of 10–20 mg/L of medium. Methyl spicifernin was obtained after methylation with diazomethane.

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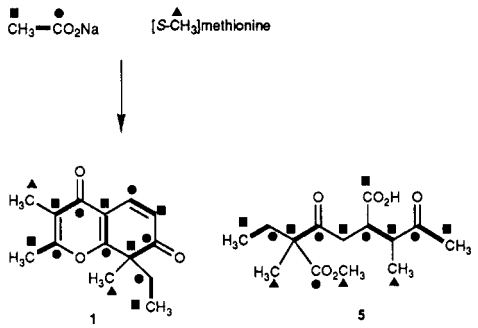
Table I. ^{13}C NMR Data[a] for Spiciferone A Enriched from Labeled Precursors

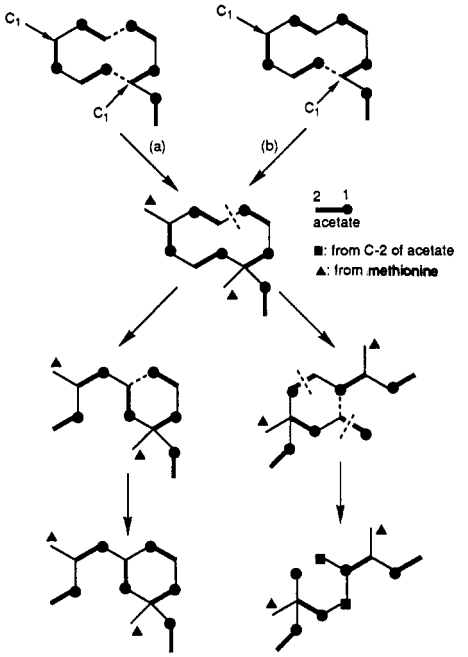
carbon	δ_C	J cc[b] (Hz)	relative enrichment [1-^{13}C]acetate	[2-^{13}C]acetate	[$^{13}CH_3$]methionine	isotopic shift[c]/ppm
2	161.0	52.7	4.0	1.0[d]	0.7	
3	120.9	54.2	1.0[d]	5.5	1.2	
4	175.4	54.2	3.3	0.8	0.8	
4a	115.5	67.9	0.9	4.5	1.2	
5	137.8	63.3	4.3	1.1	1.1	
6	123.7	63.3	0.8	4.5	1.1	
7	200.9	40.4	4.0	1.0	1.1	
8	53.3	40.4	0.7	3.8	1.0[d]	
8a	170.6	67.9	2.8	0.7	0.9	
Me-2	17.8	52.7	0.9	4.0	1.2	
Me-3	9.9		1.3	1.3	18.2	
Me-8	24.0		1.4	1.2	17.9	
CH_3CH_2-8	33.0	34.3	4.4	1.0	0.9	−0.09, −0.17, −0.26
CH_3CH_2-8	9.3	34.3	1.3	3.9	0.9	

[a] Spectra were recorded at 100.5 MHz in $CDCl_3$. [b] Coupling constants were observed in spiciferone A enriched with [1,2-$^{13}C_2$]acetate. [c] Isotopic shifts were detected in spiciferone A after incorporation of [1-^{13}C,$^2H_3$]acetate. [d] Enrichments were normalized to these signals.

Scheme I

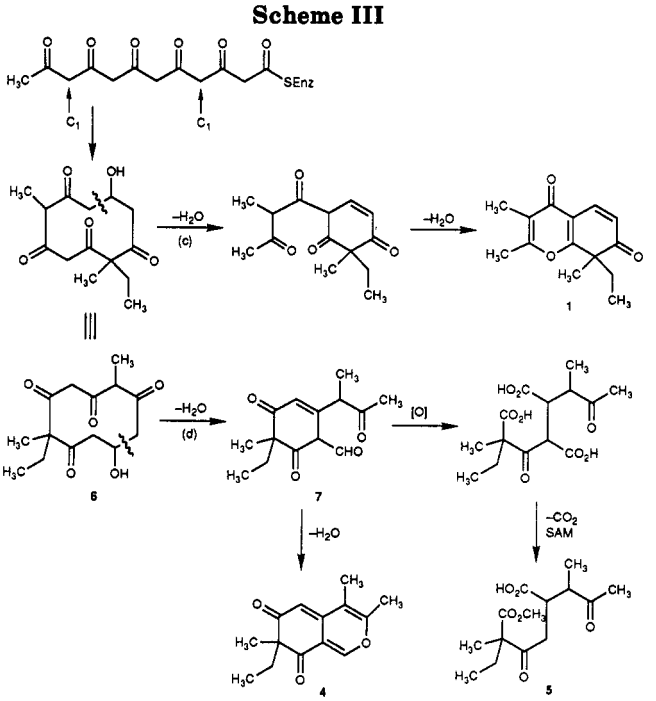


Scheme II



is subject to the C–C bond cleavage by retro-aldol condensation to produce a linear keto aldehyde intermediate, which is then recyclized and modified as in path c, spiciferone A will be formed. If the intermediate **6** is subject to the C–C bond cleavage by retro-aldol condensation, and to recyclization and dehydration as in path d, then a monocyclic intermediate **7** will be formed. The monocyclic intermediate **7** is converted into spiciferinone (**4**) by cyclization and dehydration and into spicifernin (**5**) by oxidation, and oxidative C–C bond cleavage, decarboxylation, and further introduction of a C_1 unit into the carboxyl function.

Scheme III



In a previous report,[5] we proposed the involvement of a bicyclic aromatic intermediate in the biosynthesis of spicifernin based on the retention of deuterium atoms in spicifernin in a feeding experiment with [1-^{13}C,$^2H_3$]acetate. However, deuterium atoms on the acetate precursor are sometimes easily washed out from the metabolite during the process of polyketide biosynthesis.[7-9] In addition, the involvement of a bicyclic aromatic intermediate does not account for the data with spiciferone A. Thus, we had to abandon the idea of a bicyclic aromatic intermediate in our working hypothesis. It may be worth noting that the stereochemical relationship between spiciferone A and spicifernin supports this biosynthetic route.[10] In polyketide biosynthesis, C–C bond cleavages usually occur by oxidation, including a Baeyer–Villiger reaction, and/or de-

(7) Simpson, T. J.; Stenzel, D. J. *J. Chem. Soc., Chem. Commun.* **1982**, 1074–1076.
(8) McIntyre, C. R.; Simpson, T. J.; Trimble, L. A.; Vederas, J. C. *J. Chem. Soc., Chem. Commun.* **1984**, 706–709.
(9) Lewis, C. N.; Staunton, J.; Sunter, D. C. *J. Chem. Soc., Chem. Commun.* **1986**, 58–60.

carboxylation.[11] The C–C bond cleavage by retro-aldol condensation, which is presumably operative in the biosynthesis of spiciferone A and spicifernin, is the first example of its type. Ceratenolone[12] from *Ceratocystis minor* and similin B[13] from *Sporomiella similis* are structurally similar fungal metabolites to spiciferinone. Although their biosynthesis has not been investigated yet, from their carbon skeleton they are presumably formed via the same intermediate (7) as in the biosynthesis of spiciferinone. To confirm the validity of this hypothetical pathway, a search is currently in progress for the intermediates of spiciferone A and spicifernin among the metabolites of this fungus.

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(10) The X ray crystallographical data and degradation reactions of spicifernin indicated an *S* configuration at C-7 of spicifernin. Chemical conversion of spiciferone A and application of Mosher's method[14] indicated an *R* configuration at C-8 of spiciferone A. Details will be reported elsewhere.

(11) Herbert, R. B. In *The Biosynthesis of Secondary Metabolites*, 2nd ed.; Chapman and Hall: New York, 1989; pp 31–61.

(12) Ayer, W. A.; Attah-Poku, S. K.; Browne, L. M.; Orszanska, H. *Can. J. Chem.* **1987**, *65*, 765–769.

(13) Weber, H. A.; Swenson, D. C.; Gloer, J. B. *Tetrahedron Lett.* **1992**, *33*, 1157–1160.

(14) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.