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Biosynthesis of Sorokinianin a Phytotoxin of *Bipolaris sorokiniana*: Evidence of Mixed Origin from the Sesquiterpene and TCA Pathways

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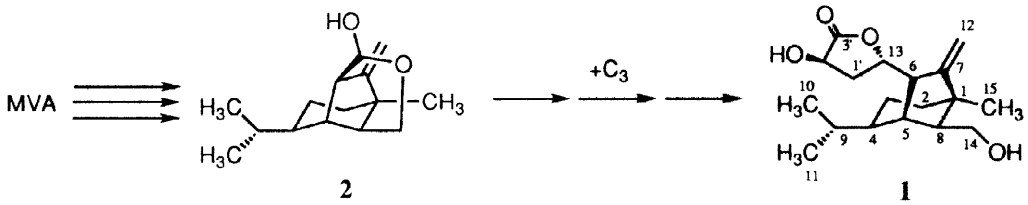
Abstract: Incorporation of sodium [1,2-$^{13}C_2$]acetate confirmed that sorokinianin is, except for the C_3 portion, a sesquiterpene and indicated that the C_3 portion is derived from the acetyl CoA-related metabolic pathway. Results of replacement culture experiments suggested that the precursor of sorokinianin is prehelminthosporol, a major metabolite of *B. sorokiniana* OB-25-1, and a TCA cycle intermediate. Incorporation of [2,3-$^{13}C_2$]succinic acid confirmed that the origin of the C_3 portion is a TCA cycle intermediate.

Bipolaris sorokiniana (Sacc.) Shoem. is a phytopathogen that causes spot blotch or foot and root rot diseases in wheat, barley, oats and other grasses.[1] During the investigation of phytopathogenic fungi as sources of and/or metabolism models for novel phytotoxic secondary metabolites,[2] we isolated a strain (OB-25-1) of *B. sorokiniana* from imported Australian barley grain (*Hordeum vulgare* L.) that produced an unknown phytotoxin which inhibited the germination of barley seeds. The isolated phytotoxin was named sorokinianin and characterized.[3] Its structure (**1**) and absolute stereochemistry were determined unambiguously by the total synthesis of this phytotoxin by H. Watanabe *et al.*[4]

Sorokinianin (**1**) is a novel fungal metabolite composed of a C_3 unit plus the same carbon skeleton as prehelminthosporol (**2**), a sesquiterpene that is the major metabolite of *B. sorokiniana* OB-25-1. There are some microbial metabolites which can be cleaved retro-biosynthetically to generate a C_3 moiety and a moiety such as fatty acid and polyketide that is assumed to be derived from an ordinary biosynthetic pathway.[5] Studies that verify the biosynthetic origin of these metabolites are limited to the fatty acid and polyketide type metabolites.[5,6] For example, avenaciolide,[7] marticin[8] and canescin[9] are derived from the polyketide and TCA pathways, and piliformic acid[10] from the fatty acid and TCA pathways; whereas, virginiae butanolide A[11] is derived from polyketide and glycerol. To our knowledge, there has been no report of the biosynthesis of a



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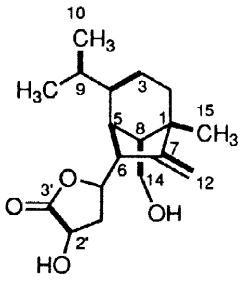

Fig.1 Labeling pattern in sorokinianin enriched with [1,2-$^{13}C_2$]acetate

metabolite, such as sorokinianin, composed of terpene portion and C_3 portion. Our purpose was to clarify the biosynthetic origin of this unique phytotoxic metabolite using stable isotope-labeled precursor-feeding experiments and replacement culture experiments.

We first conducted a sodium [1,2-$^{13}C_2$]acetate incorporation experiment to confirm the sesquiterpene origin of the carbon skeleton of sorokinianin (with the exception of the C_3 unit portion) and to explore the origin of the C_3 unit. Sodium [1,2-$^{13}C_2$]acetate was added, every 24 hr up to day 11, to 7-day-old surface cultures of *B. sorokiniana* grown on Fries medium at 24°C. Three days later the cultures were filtered, and the filtrate was extracted with EtOAc. Repeated column chromatography of the EtOAc extract and final purification by HPLC gave the labeled sorokinianin in the yield of 1.6 mg/L. Its ^{13}C NMR spectrum was measured in acetone-d_6 at 150 MHz. Results show that seven intact units of acetate were incorporated into sorokinianin and that its labeling pattern, except for the γ-lactone portion, coincided with that of the helminthosporal-type metabolite (Fig 1).[12] The labeling pattern in the γ-lactone portion suggests that that portion is derived from an acetyl-CoA related metabolic pathway that is independent of the sesquiterpene biosynthetic pathway.

We next conducted a replacement culture experiment with prehelminthosporol and the candidates for the C_3 unit precursor. Alanine was used as the pyruvate surrogate because transamination generates an endogenous pool of pyruvate. Succinic and fumaric acids were used as the TCA cycle intermediates. The fungus was grown on Fries medium in a test tube at 24°C without shaking. After 7 days the medium was replaced with phosphate buffer (pH 7.0), and the fungus cultured for 48 hr. The buffer was replaced with fresh phosphate buffer with or without the test materials and the fungus cultured for 4 days. The converted products were extracted from the culture filtrate with EtOAc. The amount of sorokinianin was determined by capillary GC-MS analysis[13] after acetylation of the sorokinianin-containing fraction obtained by purification of the extract using chromatography. A mass chromatogram monitored at *m/z* 115 and 143 was used to estimate the sorokinianin content of the fraction. Table 1 shows part of the results of the replacement culture experiments. The addition of prehelminthosporol increased the production of sorokinianin to 4 to 6 times that without prehelminthosporol. A simultaneous addition of prehelminthosporol and fumaric acid markedly stimulated sorokinianin production to 20 times that of the control. The same effect was observed for succinic

Table 1. Production of sorokinianin in replacement culture with or without prehelminthosporol (preH) and a candidate for the C_3 unit precursor

	Yield of sorokinianin (mg/L)			
Experiment (Candidate)	control	+preH	+Can	+preH, +Can
Exp. 1 (Alanine)	0.056	0.326	0.025	0.108
Exp. 2 (Succinic acid)	0.044	0.148	0.094	0.729
Exp. 3 (Fumaric acid)	0.079	0.269	0.097	1.454

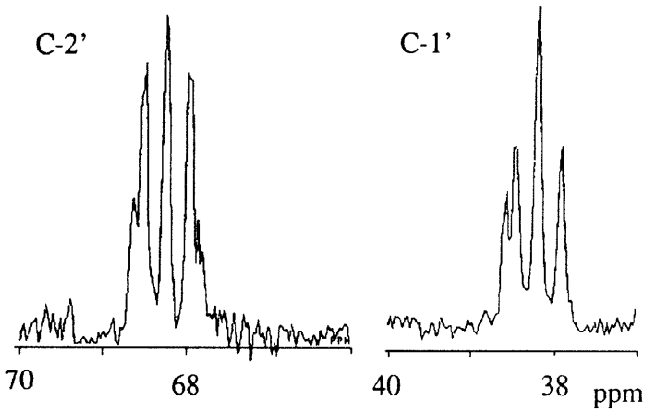

Fig. 2 ^{13}C NMR spectrum (in part, 67.8 MHz, acetone-d_6) of sorokinianin enriched with [2,3-$^{13}C_2$]succinic acid

acid, whereas alanine, serine, propionic acid and glycerol had no effect. These results suggest that the biosynthetic precursor of sorokinianin is prehelminthosporol and that the biogenetic origin of the C_3 unit portion is a member of the TCA cycle. These results agree with those of the sodium [1,2-$^{13}C_2$] acetate-feeding experiment.

To confirm our findings, we conducted a stable isotope-labeled succinic acid-feeding experiment. We measured the ^{13}C NMR spectrum of sorokinianin enriched with [2,3-$^{13}C_2$]succinic acid and found only one C-C coupling between C-1' and C-2' (Fig. 2). We therefore conclude that the biosynthetic precursor of the C_3 unit portion is a member of the TCA cycle.

Which acid of the TCA cycle is the direct sorokinianin precursor remains to be shown. Structural similarities indicate that malic acid is the most probable direct precursor. The absolute configuration of malic acid, however, does not agree with that of the C_3 unit portion of sorokinianin. The most likely candidate for the TCA cycle intermediate therefore is oxaloacetic acid, and we propose that the biosynthesis of sorokinianin from prehelminthosporol is as shown in Fig. 3. An experiment designed to verify this pathway is now in progress. Recently we isolated and characterized the cochliospicin A produced by the fungus *Cochliobolus spicifer*.[14] Cochliospicin A has a C_3 unit portion plus the same carbon skeleton as spiciferinone, a polyketide phytotoxin of that fungus. Cochliospicin A is not phytotoxic, therefore conversion of spiciferinone to cochliospicin A by a C_3 unit addition reaction causes loss of phytotoxicity by the metabolite. In contrast sorokinianin is more phytotoxic than prehelminthosporol and, interestingly, conversion of prehelminthosporol to sorokinianin results in an increase in the phytotoxicity of the metabolite.

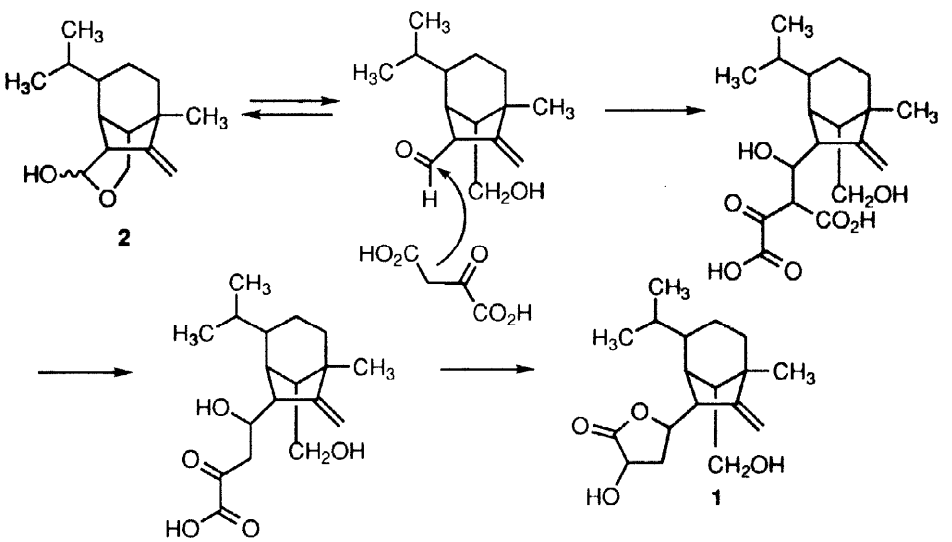

Fig. 3 Proposed biosynthesis of sorokinianin (1) from prehelminthosporol (2)

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13. GC-MS conditions: column: HP-5 (30 m, 0.32 mm i.d., 0.25 μm film thickness); oven temperature: 150°C (1 min), 15°C/min, 300°C (4 min); interface temperature: 250°C; ion source temperature: 200°C; ionization voltage: 70 eV.
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