

Isolation and Characterization of Thaxtomin-Type Phytotoxins Associated with *Streptomyces ipomoeae*

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Chromatographic investigations of *Streptomyces ipomoeae* infected potato tissue and sweetpotato tissue yielded three compounds which on aseptically cultured minitubers individually reproduced symptoms typical of the common scab disease. Spectroscopic examination of the two minor components elicited sufficient information to identify them as analogs of the major constituent which had been previously characterized as 12-*N*-methylcyclo-(L-4-nitrotryptophyl-L-phenylalanyl). Subsequent investigations of the infected tissue extracts identified a possible toxin precursor substance as 12-*N*-methylcyclo-(L-tryptophyl-L-phenylalanyl).

Keywords: *Streptomyces ipomoeae*; phytotoxins; thaxtomin C; dioxopiperazines

The actinomycete *Streptomyces ipomoeae* (Person and Martin) Waksman and Henrici is the causal agent of an economically important disease of sweetpotato, *Ipomoeae batatas* (L.) ham. Infection is characterized by substantial yield reductions and disfigured lesions on the fleshy storage roots (Person and Martin, 1940). A similar but distinct pathogen, *Streptomyces scabies* (Thaxt.) Lambert and Loria, causes common scab on several root and tuber crops, especially potatoes (Lambert and Loria, 1989). In a previous paper (King et al., 1989) we reported isolation and characterization of the phytotoxins thaxtomin A (1) and thaxtomin B (2) from potato tissue infected with pathogenic isolates of *S. scabies*. More recently, trace quantities of the thaxtomin analogs 3-5 were obtained from similarly infected sources (King et al., 1992). Our subsequent investigations examining the host-parasite relationship of pathogenic *S. ipomoeae* isolates identified the presence of three thaxtomin-type phytotoxins associated with infection of both potato and sweetpotato tissue. In this paper we describe the production and characterization of these thaxtomin analogs, investigations regarding the generality of the pathogen-toxin relationship, and the identification of a possible phytotoxin precursor.

MATERIALS AND METHODS

Chromatography. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F₂₅₄ plates and Whatman KC₁₈F plates. High-performance thin-layer chromatography (HPTLC) was performed on Whatman HP-KF plates. High-performance liquid chromatography (HPLC) utilized a Perkin-Elmer Series 4 liquid chromatograph and a Supelco RP-18 column (5- μ m particle size, 4.6 mm \times 15 cm) eluted with water/acetonitrile 75:25 (v/v) at a flow rate of 1 mL/min and monitored at 380 and 280 nm with a Shimadzu SPD-M6A photodiode array UV-vis detector.

Equipment. Infrared (IR) spectra were determined by using a Perkin-Elmer 467 grating infrared spectrophotometer.

Table 1. Summary of Isolation Data

compd	MW/formula	<i>R_f</i> values		HPLC (min)	yield (mg/100 g)
		RP-C ₁₈	silica		
3	392/C ₂₁ H ₂₀ N ₄ O ₄	0.69	0.35	7.9	<10 ⁻¹
7	378/C ₂₀ H ₁₈ N ₄ O ₄	0.74	0.26	5.6	<10 ⁻³
8	408/C ₂₁ H ₂₀ N ₄ O ₅	0.71	0.22	7.1	<10 ⁻³
9	347/C ₂₁ H ₂₁ N ₃ O ₂	0.68	0.48	6.2	<10 ⁻²

Ultraviolet (UV) spectra were recorded in absolute ethanol using a Varian Cary 219 spectrophotometer. Fast atom bombardment (FAB) and electron impact (EI) mass spectra (MS) were obtained on a Finnigan MAT 312 mass spectrometer. Xe (99.995%, Matheson) was used as the bombardment gas at 8 kV, and the resulting ions were extracted into the mass analyzer at an accelerating potential of 3 kV. All NMR spectra were recorded for solutions in deuterated methanol (unless otherwise noted) with a Varian XL-200 spectrometer operating at 200 MHz for ¹H and at 50 MHz for ¹³C. Chemical shifts were measured downfield from the signal of internal tetramethylsilane.

Phytotoxin Production and Isolation Procedures.

Isolates were maintained and subcultured on *Streptomyces* growth medium (SGM) (Clark and Lawrence, 1981) at 26 °C. Field-grown potato tubers (cv. Green Mountain) or sweetpotato storage roots (cv. Jewel) were washed, peeled, surface sterilized with hypochlorite (0.5%), and cut into thin slices (0.25-cm thickness). The slices (ca. 100 g/batch) were transferred to sterile Petri dishes and inoculated with a mycelial spore suspension of the cultured isolate. When the slices were thoroughly infected (6-7 days), they were homogenized with acetone (200 mL) to produce a fine slurry. This was filtered under vacuum through Whatman No. 1 filter paper and the filter cake washed twice with 50-mL quantities of acetone. The acetone in the combined filtrates was evaporated *in vacuo* at room temperature. The remaining aqueous suspension was diluted with 25 mL of water, transferred to a separatory funnel, and extracted twice with 150-mL portions of chloroform. The chloroform was removed *in vacuo* at room temperature and the yellow residue taken up in acetone (10 mL) and filtered to remove coextracted waxes. Most of the acetone was removed *in vacuo* and the residue fractionated by thin-

Table 2. ¹H-NMR Spectral Assignments for Compounds 3 and 7–9

hydrogen	3 ^a	7	8	9
2	7.21 s	7.10 s	6.88 s	7.06 s
4				7.64 d; <i>J</i> = 7.9
5	7.95 dd; <i>J</i> = 7.9, 1.0	7.84 dd; <i>J</i> = 7.9, 0.9	7.83 dd; <i>J</i> = 7.8, 1.0	7.10 dd; <i>J</i> = 7.9, 1.0
6	7.26 m	7.21 t; <i>J</i> = 8.0	7.20 t; <i>J</i> = 8.0	7.17 t; <i>J</i> = 8.0
7	7.65 dd; <i>J</i> = 8.1, 1.0	7.72 dd; <i>J</i> = 8.1, 0.9	7.70 dd; <i>J</i> = 8.1, 1.0	7.35 d; <i>J</i> = 8.0
10	2.93 dd; <i>J</i> = 14.5, 7.0 3.61 dd; <i>J</i> = 14.6, 4.6	2.17 dd; <i>J</i> = 14.0, 8.3 3.20 dd; <i>J</i> = 14.4, 4.4	2.08 dd; <i>J</i> = 14.4, 7.6 2.90 dd; <i>J</i> = 14.5, 5.7	3.28 dd; <i>J</i> = 14.6, 4.6 3.35 dd; <i>J</i> = 14.4, 3.3
11	4.12 dd; <i>J</i> = 7.0, 4.5	4.01 ddd; <i>J</i> = 8.0, 4.3, 1.2	3.93 dd; <i>J</i> = 7.6, 5.7	4.27 dd; <i>J</i> = 4.6, 3.2
14	4.03 tt; <i>J</i> = 9.7, 3.2, 3.0	4.12 ddd; <i>J</i> = 6.2, 4.4, 1.2		3.79 dd; <i>J</i> = 10.3, 3.2
17	1.88 dd; <i>J</i> = 13.3, 9.9 3.09 dd; <i>J</i> = 13.5, 3.8	2.48 dd; <i>J</i> = 13.6, 6.2 2.84 dd; <i>J</i> = 13.6, 4.3	2.87 d; <i>J</i> = 13.0 2.97 d; <i>J</i> = 13.0	0.68 dd; <i>J</i> = 13.5, 10.2 2.52 dd; <i>J</i> = 13.4, 3.4
19	6.84 m	7.05 m	7.12 m	6.28 m
20		7.32 m	7.33 m	7.08 m
21	} 7.17–7.31 m	7.32 m	7.33 m	7.08 m
22		7.32 m	7.33 m	7.08 m
23		7.05 m	7.12 m	6.28 m
CH ₃ (N-12)	3.09 s		2.85 s	3.12 s

^a Recorded in CDCl₃. *J* values in hertz.

layer chromatography on 0.25-mm silica gel 60 TLC plates with chloroform/methanol (9:1). Fractionated material was assayed for scab-inducing activity by appressing 4-mm antibiotic blank paper disks saturated with the material onto the surfaces of sterile minitubers (King et al., 1991). For bioactive materials lesions usually appeared within 24 h. Further purification of active from inactive material was then undertaken by fractionation on 0.2-mm RP-C₁₈ with acetone/water (3:2).

RESULTS AND DISCUSSION

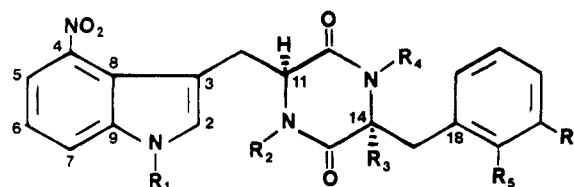
Fractionation by normal-phase TLC of the acetone-soluble extracts from both *S. ipomoeae* (isolate 78–57) infected potato and sweetpotato tissues yielded three components (one major and two minor) that when applied to aseptically cultured minitubers individually reproduced symptoms typical of the common scab disease. These bioactive materials were then subjected to further cleanup by reversed-phase TLC. This procedure subsequently furnished chromatographically (HPTLC and HPLC) homogeneous entities that were yellow and exhibited relatively similar UV, IR, and MS characteristics. Table 1 summarizes isolation and molecular weight data.

The major component, now provisionally called thaxtomin C and reported previously (King et al., 1992), was characterized predominantly on the basis of ¹H-NMR methodologies utilized in the structural determinations of thaxtomins A and B (King et al., 1992). For example, comparison of its ¹H-NMR and ¹H homonuclear shift correlated (COSY) spectra with that of *N*-acetyl-4-nitrotryptophan and phenylacetic acid facilitated complete proton assignments for the aromatic hydrogens (Table 2). Subsequent determination (via ¹H-COSY spectra) of an allylic coupling (*J* < 0.5 Hz) between H-2 of the 4-nitroindol-3-yl group and a methylenic proton at δ 3.61 (H-10) allowed complete assignment of the aliphatic protons for thaxtomin C (Table 2). The preferred conformation (in solution) of thaxtomin C can also be inferred from analysis of its ¹H-NMR data. Hence, the fact that one of the H-17 protons is substantially shielded (i.e., shifted upfield relative to linear peptides containing phenylalanine) indicates that in thaxtomin C the tryptophan moiety is folded over the dioxopiperazine ring (Maes et al., 1986). The penultimate step in definition of the structure 3 for thaxtomin C involved assignment of an *N*-methyl substituent to the N-12 position on the basis of an observed coupling

Table 3. ¹³C-NMR Spectral Assignments for Compound 3

carbon	δ	multiplicity
2	130.70	d
3	108.40	s
4	142.18	s
5	117.17	d
6	119.84	d
7	115.05	d
8	118.32	s
9	138.89	s
10	32.59	t
11	62.75	d
13	165.26 ^a	s
14	55.95	d
16	165.54 ^a	s
17	38.99	t
18	136.62	s
19	129.61	d
20	128.22	d
21	126.52	d
22	128.22	d
23	129.61	d
CH ₃ (N-12)	29.70	q

^a Assignments may be reversed.

**Figure 1.** Structural formulas of compounds 1–8.

compd	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
1	H	Me	OH	Me	H	OH
2	H	Me	OH	Me	H	H
3	H	Me	H	H	H	H
4	H	Me	OH	Me	OH	H
5	H	Me	H	Me	H	H
6	Me	Me	H	Me	H	H
7	H	H	H	H	H	H
8	H	Me	OH	H	H	H

between H-14 and an adjacent amide proton in the ¹H-COSY spectra. The structural elucidation of this compound was further substantiated by a direct comparison of its *N,N,N*-trimethyl derivative with a synthetic sample of 1,12,15-*N,N,N*-trimethylcyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (6) (King et al., 1992). A ¹³C-NMR spectrum (Table 3) compatible with the assigned structure was also recorded.

Table 4. Origin of *Streptomyces* Isolates and Pathogenicity Assay Results

species assignment	strain no.	location	isolated by	scab symptoms on minitubers	production of thaxtomin C	pathogenicity on sweetpotato
<i>S. ipomoeae</i>	78-58	North Carolina	J. Moyer	+	+	+
<i>S. ipomoeae</i>	81-45	California	C. Clark	+	+	+
<i>S. ipomoeae</i>	ATCC-25462	Louisiana	E. Shirling	-	-	-
<i>S. ipomoeae</i>	81-44	Louisiana	C. Clark	+	+	+
<i>S. ipomoeae</i>	78-60	North Carolina	J. Moyer	+	+	+
<i>S. ipomoeae</i>	78-61	North Carolina	J. Moyer	+	+	+
<i>S. ipomoeae</i>	78-62	North Carolina	J. Moyer	+	+	+
<i>S. ipomoeae</i>	91-3	North Carolina	J. Ristaino	+	+	+
<i>S. species</i>	T-9	North Carolina	J. Ristaino	-	-	-
<i>S. galbus</i>	ATCC-23910	Hungary	G. Szakaes	-	-	-

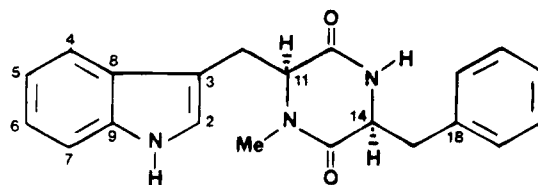
Compound **7** (Figure 1), although never before isolated from natural sources, proved identical (UV, IR, MS, and $^1\text{H-NMR}$) to a synthetic sample of cyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (**7**) prepared in a previous study (King et al., 1992).

Compound **8**, the most complex component of the mixture, was formulated as a monooxygenated analog of thaxtomin C (**3**) on the basis of its molecular weight. A comparative analysis of its $^1\text{H-COSY}$ and $^1\text{H-NMR}$ spectra (Table 2) with that of thaxtomin C (**3**) then established the structure of this compound as 12-*N*-methyl-14-hydroxycyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (**8**). Assignment of a hydroxyl substituent to the C-14 position was determined on the evident lack of a proton therein.

From assessment of the foregoing structural determinations it is apparent that cyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (**7**) now represents the most fundamental member of the thaxtomin group yet identified. Accordingly, this compound also represents the entity from which other members of the group (i.e., compounds **3** and **8**) generated by *S. ipomoeae* are most likely derived.

To help ascertain the generality of the pathogen-toxin relationship, a total of 10 *Streptomyces* strains with predetermined pathogenicity on sweetpotato were assayed for any associated effect on aseptically cultured potato minitubers and the production of thaxtomin-type phytotoxins on infected tuber tissue. As outlined in Table 4, only the strains predetermined to be pathogenic on sweetpotato caused scablike symptoms on potato minitubers and the production of thaxtomin C on infected tissue (both potato and sweetpotato). As in the case of *S. scabies*, whether production of the toxin is a prerequisite or a byproduct of pathogenicity has still to be determined.

In a continuing effort to ascertain the biosynthetic sequence of events in the production of these phytotoxins, we undertook a search for the possible presence of any unnitrated dioxopiperazines in the *S. ipomoeae* infected potato extracts (earlier attempts to detect 4-nitrotryptophan precursors were unsuccessful). In these endeavors utilization of a HPLC diode array system was instrumental in first detecting the presence of a suspected unnitrated dioxopiperazine compound. Subsequently, we were successful in isolating via TLC procedures (see Table 1 and Materials and Methods for TLC parameters) relevant quantities of the candidate compound. Mass spectrometry combined with utilization of a $^1\text{H-NMR}$ regimen similar to that outlined for the characterization of thaxtomin C (**3**) (see Tables 1 and 2) identified the compound as 12-*N*-methylcyclo-(L-tryptophyl-L-phenylalanyl) (**9**), (Figure 2) an unnitrated analog of the major phytotoxin (**3**) from *S. ipomoeae*. From these findings, it is tempting to conclude that nitration is the final step (whether through

**Figure 2.** Structural formula of compound **9**.

the normal sequence of $-\text{OH} \rightarrow \text{NH}_2 \rightarrow \text{NO}_2$ or direct nitration) in the formation of the *S. ipomoeae* phytotoxin (**3**). However, the possibility also exists that the unnitrated dioxopiperazine (**9**) simply represents a portion of material that somehow evaded an earlier nitration procedure.

Of interest in our attempt to correlate production of the thaxtomins by *S. ipomoeae* and *S. scabies* isolates were the findings that pathogenic *S. ipomoeae* isolates did not generate phytotoxins when grown on any of the usual growth media (i.e., oatmeal broth, tryptic soy, YGM, SGM, etc.). These findings are in direct contrast to the results obtained with pathogenic *S. scabies* isolates grown on oatmeal broth (Babcock et al., 1993) and may indicate some divergence in the respective biosynthetic pathways.

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