Characterization of New Thaxtomin A Analogues Generated *in Vitro* by *Streptomyces scabies*

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Examination of the phytotoxic exudates associated with the *in vitro* production of thaxtomin A by *Streptomyces scabies* yielded four new members of this class of phytotoxins. The new compounds were characterized by spectral means as the *p*-hydroxyphenyl (**7**), 3,4-dihydroxyphenyl (**8**), 15-de-*N*-methyl (**9**), and 12-de-*N*-methyl (**10**) analogues of thaxtomin A (**1**).

Keywords: *Streptomyces scabies; phytotoxins; thaxtomins*

Common scab of potato, generally attributed to infection by the soil bacterium *Streptomyces scabies* (Thaxt.) Lambert and Loria, is considered a disease of major economic importance in most potato-producing areas of the world (Lambert and Loria, 1989a). A less common though still important cause of potato scab, *Streptomyces acidiscabies* Lambert and Loria, is operative in acidic soils (Lambert and Loria, 1989b). Our *in vivo* host interaction studies involving both *S. scabies* and *S. acidiscabies* determined that phytotoxins may play an important role in the pathogenicity of these organisms (King et al., 1991). The phytotoxins named thaxtomins (in honor of Roland Thaxter, the American plant pathologist who first identified the causal organism of common scab) consist of 4-nitroindol-3-yl-containing 2,5-dioxopiperazines (King et al., 1989). While thaxtomin A (**1**) was determined to be the predominant phytotoxin associated with both *S. scabies* and *S. acidiscabies*, minor amounts of four other related compounds have also been isolated and characterized (King et al., 1992). The opportunate finding that both organisms could be induced to generate the phytotoxins *in vitro* (Babcock et al., 1993; Loria et al., 1995) provided an improved venue for determining intermediates in the phytotoxin biosynthetic scheme, and in a recent paper (King et al., 1995) we reported the identification of *N*-acetyl- and *N*-methyl-4-nitrotryptophan produced in association with the *in vitro* production of thaxtomin A by *S. scabies*. We now report the isolation and characterization of four new members of the thaxtomin class of phytotoxins generated *in vitro* by *S. scabies* and speculate on their probable place in the biosynthetic scheme.

MATERIALS AND METHODS

Chromatography. Thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} plates and Whatman $KC_{18}F$ plates. High-performance thin-layer chromatography (HPTLC) was performed on Whatman HP-KF plates.

Equipment. Ultraviolet (UV) spectra were recorded in absolute ethanol using a Varian Cary 219 spectrophotometer. Chemical ionization (CI) and electron impact (EI) mass spectra (MS) were obtained on a Finnigan MAT 312 mass spectrometer. All NMR spectra were recorded for solutions in deuterated methanol (unless otherwise noted) with a Varian Unity 400 operating at 400 MHz for ^{1}H. Chemical shifts were measured downfield from the signal of internal tetramethylsilane.

Phytotoxin Production and Isolation Procedures. Isolates were maintained and subcultured on solid modified glucose medium as detailed previously (King et al., 1991).

Table 1. Summary of Isolation Data

compd	MW/formula	R_f values: RP-C_{18}	R_f values: silica	yield (mg/100 mL)
1	438/$C_{22}H_{22}N_4O_6$	0.76	0.27	<0.5
2	438/$C_{22}H_{22}N_4O_6$	0.72	0.29	$<10^{-2}$
3	392/$C_{21}H_{20}N_4O_4$	0.69	0.35	$<10^{-3}$
4	422/$C_{22}H_{22}N_4O_5$	0.66	0.41	$<10^{-2}$
5	406/$C_{22}H_{22}N_4O_4$	0.62	0.45	$<10^{-3}$
6	408/$C_{21}H_{20}N_4O_5$	0.71	0.23	$<10^{-3}$
7	438/$C_{22}H_{22}N_4O_6$	0.79	0.21	$<10^{-3}$
8	454/$C_{22}H_{22}N_4O_7$	0.82	0.18	$<10^{-2}$
9	424/$C_{21}H_{20}N_4O_6$	0.77	0.13	$<10^{-3}$
10	424/$C_{21}H_{20}N_4O_6$	0.77	0.15	$<10^{-3}$

Oatmeal broth medium was prepared by boiling 40 g of oatmeal/800 mL of water for 5 min in a microwave oven. The broth was cooled to approximately 50 °C and filtered through a fine mesh cheesecloth. The filtrate was adjusted to 1 L with distilled water, and 2.0 mg of $ZnSO_4 \cdot 7H_2O$ was added. After the pH was adjusted to 6.8 with 0.1 N NaOH, 100 mL portions of the medium were dispensed into 500 mL flasks and sterilized at 15.0 lb for 20 min. The oatmeal medium was then inoculated with 5 mL of a 3-day-old shake culture of the test organism. The cultures were then incubated at 26 °C on a rotary shaker. At maximum phytotoxin production (4–5 days), the cell cultures were transferred to a separatory funnel and extracted twice with 150 mL portions of ethyl acetate. The ethyl acetate extracts were dried over anhydrous sodium sulfate, and the ethyl acetate was removed *in vacuo* at 25 °C. The residue was taken up in acetone and fractionated on 0.25 mm silica gel 60 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 mini-tubers (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-C_{18} plates with acetone/water (3:2).

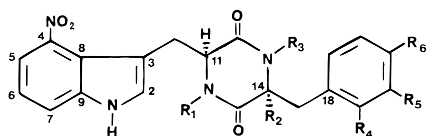
RESULTS AND DISCUSSION

Fractionation by silica gel TLC of the ethyl acetate soluble extracts from oatmeal broth cultures of pathogenic *S. scabies* isolates (King et al., 1991) yielded up to 10 yellow components (1 major and 9 minor) that when applied to aseptically cultured mini-tubers individually reproduced symptoms typical of the common scab disease. These bioactive materials were then subjected to further cleanup by reversed-phase TLC. This procedure furnished chromatographically (HPTLC) homogeneous compounds that exhibited relatively similar UV, MS, and ^{1}H-NMR characteristics. Table 1

0021-8561/96/1444-1108$12.00/0 Published 1996 by the American Chemical Society

Table 2. ¹H-NMR Spectral Assignments for Compounds 7−10

H	7	8	9	10
2	6.88 s	7.03 s	6.99 s	7.05 s
5	7.84 dd, $J = 7.9, 1.0$ Hz	7.84 dd, $J = 7.9, 1.0$ Hz	7.83 dd, $J = 7.9, 1.0$ Hz	7.82 dd, $J = 8.0, 1.0$ Hz
6	7.19 t, $J = 8.0$ Hz	7.19 t, $J = 8.0$ Hz	7.19 t, $J = 8.0$ Hz	7.19 t, $J = 8.0$ Hz
7	7.70 dd, $J = 8.1, 1.0$ Hz	7.70 dd, $J = 8.0, 1.0$ Hz	7.69 dd, $J = 8.0, 1.0$ Hz	7.70 dd, $J = 8.0, 1.0$ Hz
10	1.82 dd, $J = 14.1, 8.9$ Hz	2.07 dd, $J = 14.1, 8.2$ Hz	1.92 dd, $J = 14.2, 8.7$ Hz	1.20 dd, $J = 13.5, 10.2$ Hz
	2.64 dd, $J = 14.1, 6.0$ Hz	2.66 dd, $J = 14.1, 6.8$ Hz	2.75 dd, $J = 14.2, 6.5$ Hz	3.03 dd, $J = 13.6, 4.1$ Hz
11	3.87 dd, $J = 8.8, 6.0$ Hz	3.90 dd, $J = 8.1, 6.8$ Hz	3.87 dd, $J = 8.7, 6.5$ Hz	3.94 ddd, $J = 10.3, 4.1, 2.4$ Hz
17	3.06 d, $J = 14.0$ Hz	3.09 d, $J = 13.5$ Hz	3.13 d, $J = 13.5$ Hz	3.16 d, $J = 13.5$ Hz
	3.20 d, $J = 14.0$ Hz	3.39 d, $J = 13.5$ Hz	3.20 d, $J = 13.5$ Hz	3.25 d, $J = 13.5$ Hz
19	6.83 d, $J = 8.4$ Hz	6.61 d, $J = 2.9$ Hz	6.73 m	6.71 m
20	7.02 d, $J = 8.4$ Hz			
21			6.65 d, $J = 8.0$ Hz	6.67 d, $J = 8.0$ Hz
22	7.02 d, $J = 8.4$ Hz	6.70 d, $J = 8.5$ Hz	7.23 t, $J = 8.0$ Hz	7.23 t, $J = 8.0$ Hz
23	6.83 d, $J = 8.4$ Hz	6.58 dd, $J = 8.5, 3.1$ Hz	6.73 m	6.75 dd, $J = 8.0, 2.5$ Hz
CH_3 (N-12)	2.81 s	2.78 s	2.88 s	
CH_3 (N-15)	2.99 s	3.00 s		3.13 s



Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	Me	OH	Me	H	OH	H
2	Me	OH	Me	OH	H	H
3	Me	H	H	H	H	H
4	Me	OH	Me	H	H	H
5	Me	H	Me	H	H	H
6	Me	OH	H	H	H	H
7	Me	OH	Me	H	H	OH
8	Me	OH	Me	H	OH	OH
9	Me	OH	H	H	OH	H
10	H	OH	Me	H	OH	H

Figure 1. Structural formulas of compounds **1**−**10**.

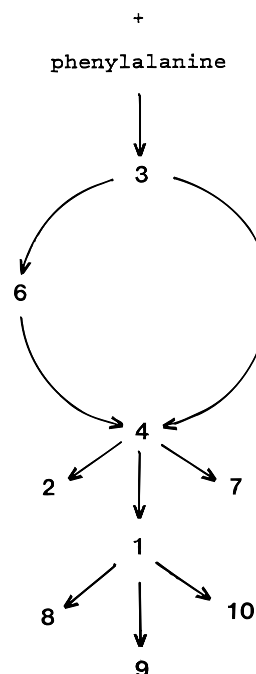


Figure 2. Proposed sequence for biosynthesis of the thaxtomins.

summarizes isolation and molecular weight data. On the basis of a comparative analysis of their ¹H-NMR, MS, and chromatographic data, five of these compounds (including the major one), i.e., compounds **1**−**5** (Figure 1), were readily identifiable as the thaxtomins isolated from *S. scabies* infected potato slices (King et al., 1992). A sixth compound, again on the basis of comparative chromatographic and spectral data, was identical to the thaxtomin phytotoxin (**6**) isolated from *Streptomyces ipomoeae* infected potato or sweet potato slices (King et al., 1994). MS revealed that compound **7** was of the same composition and had a fragmentation pattern similar to that of thaxtomin A (**1**). Subsequent assignment of the structure for compound **7** as the *p*-benzyloxy isomer of thaxtomin A was based on ¹H COSY spectra (Table 2) and comparative studies with samples of *p*-hydroxyphenylacetic acid and thaxtomin A (**1**).

Compound **8**, the most complex component of the mixture, was formulated as a monooxygenated derivative of thaxtomin A (**1**) on the basis of its molecular weight. Ultimate assignment of the structure for compound **8** as the 3,4-dihydroxyphenyl analogue of thaxtomin A (**1**) was based on its ¹H COSY spectra and comparative studies with samples of 3,4-dihydroxyphenylacetic acid and thaxtomin A (**1**).

MS and ¹H-NMR spectral analysis of the two remaining compounds indicated them to be de-*N*-methyl analogues of thaxtomin A (**1**). Characterization of the most polar of the two isomers as the 15-de-*N*-methyl analogue (**9**) relied primarily upon a comparative analysis of its MS and ¹H COSY spectra with the 12-*N*-methyl compound **7** described previously. The validity of the preceding assignment was later substantiated on the basis of an observed coupling between H-11 and an adjacent amide proton when a ¹H-NMR spectrum of the alternate isomer (**10**) was recorded in deuterated dimethyl sulfoxide.

Our initial surprise in detecting a de-12-*N*-methyl analogue (**10**) of thaxtomin A (since related precursors have not been isolated) was tempered by the subsequent finding that this compound was generated during the latter stages of phytotoxin production. Thus, it is probable that an oxidative N-demethylation of thaxtomin A (**1**) is solely responsible for the production of compound **10** and also possibly of compound **9**.

On the basis of the foregoing structural elucidations and previous subsidiary determinations (King et al., 1994, 1995; Babcock et al., 1993) a possible sequence of

events relating to formation of the thaxtomin group of phytotoxins is outlined in Figure 2. This representation projects two possible pathways to a common intermediate preceding formation of the higher order thaxtomins. Preference for one route over the other may well depend on spacial hindrances; i.e., oxygenation at C-14 may be less hindered prior to N-methylation at N-15 or vice versa.

ACKNOWLEDGMENT

We thank Peter Penner and Larry Calhoun, University of New Brunswick, Fredericton, for the NMR recordings and Pierre Lapointe of the Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa, ON, for the mass spectral determinations.

LITERATURE CITED

Babcock, M. J.; Eckwall, E. C.; Schottel, J. L. Production and regulation of potato-scab-inducing phytotoxins by *Streptomyces scabies*. *J. Gen. Microbiol.* **1993**, *139*, 1579−1586.

King, R. R.; Lawrence, C. H. Characterization of 4-nitrotryptophans associated with the *in vitro* production of thaxtomin A by *Streptomyces scabies*. *Phytochemistry* **1995**, *40* (1), 41−43.

King, R. R.; Lawrence, C. H.; Clark, M. C.; Calhoun, L. A. Isolation and characterization of phytotoxins associated with *Streptomyces scabies*. *J. Chem. Soc., Chem. Commun.* **1989**, *13*, 849−850.

King, R. R.; Lawrence, C. H.; Clark, M. C. Correlation of phytotoxin production with pathogenicity of *Streptomyces scabies* isolates from scab infected tubers. *J. Am. Potato Assoc.* **1991**, *68*, 675−680.

King, R. R.; Lawrence, C. H.; Calhoun, L. A. Chemistry of phytotoxins associated with *Streptomyces scabies*, the causal organism of potato common scab. *J. Agric. Food Chem.* **1992**, *40*, 834−837.

King, R. R.; Lawrence, C. H.; Calhoun, L. A.; Ristaino, J. B. Isolation and characterization of thaxtomin-type phytotoxins associated with *Streptomyces ipomoeae*. *J. Agric. Food Chem.* **1994**, *42*, 1791−1794.

Lambert, D. H.; Loria, R. *Streptomyces scabies* sp. nov. nom. rev. *Int. J. Syst. Bacteriol.* **1989a**, *39*, 387−392.

Lambert, D. H.; Loria, R. *Streptomyces acidiscabies* sp. nov. *Int. J. Syst. Bacteriol.* **1989b**, *39*, 393−396.

Loria, R.; Bukhalid, R. A.; Creath, R. A.; Leiner, R. H.; Olivier, M.; Steffens, J. C. Differential production of thaxtomins by pathogenic *Streptomyces* species *in vitro*. *Phytopathology* **1995**, *85*, 537−541.

Received for review April 24, 1995. Revised manuscript received July 28, 1995. Accepted February 6, 1996.[⊗]

JF950243O

[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1996.