Isolation and Identification of Pigments Generated *in Vitro* by *Streptomyces acidiscabies*

Russell R. King* and C. Harold Lawrence

Agriculture and Agri-Food Canada, Research Centre, P.O. Box 20280, Fredericton, New Brunswick, Canada E3B 4Z7

Larry A. Calhoun

Department of Chemistry, University of New Brunswick, Fredericton, New Brunswick, Canada E3B 6E2

Examination of the nonphytotoxic exudates associated with the *in vitro* production of thaxtomin A by *Streptomyces acidiscabies* yielded three pigments. The pigments were identified by chromatographic and spectral means as unusual naphthoquinone derivatives.

Keywords: *Streptomyces acidiscabies; naphthoquinones, Streptomyces auranticolor*

INTRODUCTION

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* indicated that phytotoxins may play an important role in the pathogenicity of these organisms (King et al., 1991). Thaxtomin A was determined to be the predominant phytotoxin associated with both *S. scabies* and *S. acidiscabies* (King et al., 1989, 1991), but minor amounts of other analogs have also been isolated and characterized (King et al., 1992, 1996). The opportune discovery that both organisms could be induced to generate the phytotoxins *in vitro* (Babcock et al., 1994, Loria et al., 1995) provided an improved venue for determining intermediates in the phytotoxin biosynthetic scheme (King et al., 1995, 1996).

During our *in vitro* studies of the production of thaxtomin A we also noted some corresponding generation of pigments by the *S. acidiscabies* strains. The chemical nature of these pigments had not previously been defined (Lambert and Loria, 1989b), so we undertook to purify and characterize them in an effort to determine whether they might constitute distinguishing markers for *S. acidiscabies*.

MATERIALS AND METHODS

Chromatography. Thin-layer chromatography (TLC) was performed on 0.5-mm Merck silica-gel 60 F_{254} and 0.2-mm Whatman $KC_{18}F$ plates.

Equipment. Infrared (IR) spectra were determined with a Perkin-Elmer 467 grating IR spectrophotometer. Ultraviolet (UV) spectra were recorded in absolute ethanol with a Varian Cary 219 spectrophotometer. Chemical-ionization (CI) and electron-impact (EI) mass spectra (MS) were obtained on a Finnigan MAT 312 mass spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded for solutions in deuterated methanol (unless otherwise noted) with a Varian Unity 400 spectrometer operating at 400 MHz for ^1H and at 100 MHz for ^{13}C NMR.

Table 1. Summary of Isolation Data

compd	MW/formula	R_f values: silica	R_f values: RP-C_{18}	av yield (mg/100 mL)
1	338/$C_{19}H_{14}O_6$	0.33	0.85	6.3
2	354/$C_{19}H_{14}O_7$	0.19	0.91	2.8
3	336/$C_{19}H_{12}O_6$	0.86	0.28	0.2

Pigment Production and Isolation Procedures. Scab-inducing *Streptomyces* isolates (King et al., 1991) and strains of *S. acidiscabies* (ATCC 49003, ATCC 49004, and RL 182) were maintained and subcultured on solid-modified glucose medium as detailed previously (King et al., 1991). *Streptomyces* growth medium (SGM) contained mannitol (20.0 g), K_2HPO_4 (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (5.0 g), $CaCO_3$ (2.0 g), CoCl (1.0 mL of a 110 mg/L stock solution), yeast extract (1.0 g), and agar (18.0 g) in distilled water (1000.0 mL). One hundred-milliter portions of the medium were dispensed into 500-mL flasks and sterilized at 15.0 lb for 20 min. The medium was then inoculated with 5 mL of a 3-day-old shake culture of the test organism. The cultures were then incubated at 29 °C on a rotary shaker. At maximum pigment production (5 to 6 days), the cell cultures were transferred to a separatory funnel, made acidic with 2 N HCl, 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 under reduced pressure at 25 °C. The residue was taken up in acetone and fractionated on silica-gel TLC plates with benzene:methanol (4:1). Further purification of pigmented materials was undertaken on C_{18} reversed-phase TLC plates developed in acetone:water (11:9).

RESULTS AND DISCUSSION

Our initial isolation of pigments associated with cultures of *Streptomyces acidiscabies* proceeded from an extended search for potential intermediates in the biosynthesis of thaxtomin A. In those instances, it was noted how fractionation on silica-gel TLC of the ethyl acetate extracts from oatmeal broth cultures of *S. acidiscabies* consistently yielded minor amounts of two nonphytotoxic, colored materials. To obtain these pigments in amounts sufficient for structural determinations, alternate growth media for their generation were investigated. When cultured on SGM, *S. acidiscabies* produced substantial quantities of both pigments without any cogeneration of the thaxtomins.

* Author to whom correspondence should be addressed.

S0021-8561(95)00766-7 CCC: $12.00 Published 1996 by the American Chemical Society

Table 2. ^{1}H and ^{13}C NMR Spectra Assignments for Compounds 1−3

	1[a,c]		**2**[a,c]		**3**[b,d]	
carbon	^{13}C mult	^{1}H *J* (Hz)	^{13}C mult	^{1}H *J* (Hz)	^{13}C	^{1}H *J* (Hz)
1	184.86 s		183.38 s		180.46 s	
2	152.68 s		140.49 s		148.67 s	
3	135.43 d	6.72 s	167.53 s		156.86 s	
4	192.01 s		191.69 s		192.00 s	
5	162.28 s		162.36 s		160.13 s	
6	124.37 d	7.24 dd (8.3, 1.0)	121.96 d	7.05 dd (8.2, 1.2)	121.21 d	7.32 bd (8.3)
7	137.07 d	7.64 dd (8.2, 7.5)	137.48 d	7.54 dd (8.2, 7.5)	137.12 d	7.75 t (7.8)
8	120.12 d	7.57 dd (7.5, 1.0)	119.10 d	7.48 dd (7.5, 1.2)	120.02 d	7.47 bd (7.2)
9	134.57 s		136.96 s		134.22 s	
10	116.68 s		116.09 s		117.88 s	
1′	121.23 s		121.54 s		121.84 s	
2′	158.15 s		159.13 s		158.98 s	
3′	114.31 d	6.96 s	114.45 d	6.88 s	113.87 d	7.51 s
4′	141.24 s		138.95 s		144.21 s	
5′	123.30 d	7.34 s	122.52 d	7.16 s	123.36 d	7.58 s
6′	119.75 s		120.41 s		118.45 s	
OCH3	56.52 q	3.75 s	56.38 q	3.70 s	56.64 q	3.88 s
CH3	21.76 q	2.40 s	21.65 q	2.37 s	21.43 q	2.48 s
COOR	169.86 s		175.51 s		179.78 s	

[a] Assignments based on COSY, HMQC and HMBC experiments; multiplicities based on DEPT experiments; the letters after the values refer to singlet, doublet, triplet, and quadruplet. [b] Assignments based on comparison with **1** and **2**. [c] Chemical shifts in CD_3OD, ∂CD_3OD, 49.0 ppm, ^{13}C; 3.30 ppm, ^{1}H. [d] Chemical shifts in $(CD_3)_2SO$, $\partial(CD_3)_2SO$, 39.5 ppm, ^{13}C; 2.49 ppm, ^{1}H.

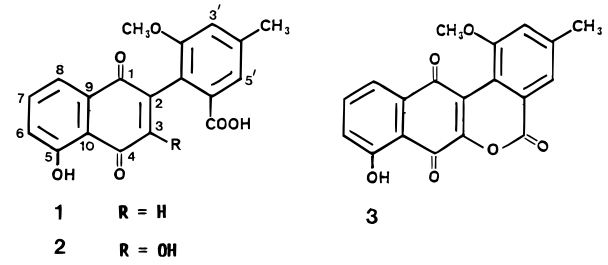

Figure 1. Structural formulas of **1**−**3**.

Isolation of the pigments generated on SGM medium again involved fractionation of the ethyl acetate extracts on silica-gel TLC plates. This procedure furnished large quantities of a bright yellow compound (**1**) and lesser amounts of a more polar orange one (**2**). It was later observed that prolonging the generation time resulted in an increased yield of the orange component at the expense of the yellow. Both crude materials were further purified by reversed-phase TLC. The isolation data are summarized in Table 1.

The molecular formula of **1** was deduced to be $C_{19}H_{14}O_6$ from high-resolution MS and hybridized carbon signals observed in DEPT ^{13}C NMR experiments [2sp^3 (2q) and 17 sp^2 (11s and 6d)] (Table 2). The ^{1}H NMR results indicated the presence of 12 unexchangeable protons, six of which were of an aromatic or alkenic nature (Table 2).

In an analogous manner, the molecular formula of **2** was deduced to be $C_{19}H_{14}O_7$ (see Table 2).

A search of the chemical literature for previously identified chemicals resembling the two pigments revealed that Ikushima et al. (1980b) had previously characterized three unusual naphthoquinones (generated by a soil microorganism provisionally named *Streptomyces auranticolor*), two of which had spectral and chromatographic properties identical to those of **1** and **2** (Figure 1). The third naphthoquinone identified by Ikushima et al. (1980b) is a dehydration conversion product of **2** that is designated **3** (Figure 1). A resurvey of our fractionated extracts subsequently confirmed the presence of **3** but in very limited amounts. This group of pigments was described as unique in that **3** was the first reported example of a natural product possessing a 5*H*-benzo[*d*]naptha[2,3-*b*]pyran ring system.

A comparison of the published morphological and physiological characteristics for *S. auranticolor* (Ikushima et al., 1980a) and *S. acidiscabies* (Lambert and Loria, 1989b) indicate that they are very similar if not identical species. Although Ikushima et al. (1980b) did not report any generation of thaxtomin-like materials when *S. auranticolor* was cultured under appropriate conditions, their investigations were focused on antibiotic production and unrelated substances may have been overlooked. Unfortunately, a culture of *S. auranticolor* was not readily available for thaxtomin production assessment, and this aspect of the study remains unresolved.

An interesting outcome from characterization of the pigments associated with *S. acidiscabies* is the documented antibiotic activity of **1** and **3** (Ikushima et al., 1980b). Thus, in addition to producing phytotoxins geared to tuber incursions, *S. acidiscabies* is also capable of erecting a chemical defense against other competing microorganisms.

We have now examined >60 scab-inducing *Streptomyces* isolates (from five different countries) and three strains of *S. acidiscabies* for their ability to produce both thaxtomin A and **1**, **2**, and **3** *in vitro*. All these organisms produced identifiable amounts of thaxtomin A, but only the *S. acidiscabies* specimens also generated measurable quantities of **1**, **2**, and **3**. These results, although limited in scope, project a promising potential for the utilization of **1**, **2**, and **3** as distinguishing markers for *S. acidiscabies* isolates.

ACKNOWLEDGMENT

We thank Pierre Lafontaine of the Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa, for the mass spectral data and Dr. Rosemary Loria, Cornell University, Ithaca, NY, for one of the *S. acidiscabies* strains.

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.

Ikushima, H.; Iguchi, I.; Kohsaka, M.; Aoki, H.; Imanaka, H. *Streptomyces auranticolor* sp. nov., a new anticoccidial antibiotics producer. *J. Antibiot.* **1980a**, *33*, 1103−1106.

Ikushima, H.; Okamoto, M.; Tanaka, H.; Ohe, O.; Kohsaka, M.; Aoki, H.; Imanaka, H. New anticoccidial antibiotics, W5-5995 A and B. Isolation and characterization. *J. Antibiot.* **1980b**, *33*, 1107−1113.

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. 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. Characterization of new thaxtomin A analogs generated *in vitro* by *Streptomyces scabies*. *J. Agric. Food Chem.* **1996**, *44*, 1108−1110.

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 November 20, 1995. Revised manuscript received May 10, 1996. Accepted June 19, 1996.[⊗]

JF950766W

[⊗] Abstract published in *Advance ACS Abstracts*, August 1, 1996.