Microbial Glucosylation of Thaxtomin A, a Partial Detoxification

Russell R. King* and C. Harold Lawrence

Agriculture and Agri-Food Canada, Potato 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

Thaxtomin A (**1**) and B (**2**), the two major phytotoxins associated with the common scab of potato disease, were transformed into C-14 linked β-glucosides (**3**) and (**4**), respectively, when individually incubated with cultures of *Bacillus mycoides* in oatmeal broth at 26 °C. These biotransformation products when assayed on aseptically produced potato minitubers proved to be much less phytotoxic than the parent compounds.

Keywords: *Thaxtomin A; phytotoxins; glucosylation; detoxification; Streptomyces scabies; Bacillus mycoides*

INTRODUCTION

Investigations of phytotoxins generated by *Streptomyces scabies* (Thaxt.) Lambert and Loria (the main causal organism of potato common scab) (Loria et al., 1997) have resulted in the isolation and characterization of a series of unique 4-nitroindol-3-yl-containing 2,5-dioxopiperazines typified by the predominant representative thaxtomin A (**1**) (King et al., 1989, 1992; King and Lawrence, 1996).

A positive correlation between the relative susceptibilities of potato cultivars to *S. scabies* and their corresponding sensitivities to thaxtomin A has also been established (Delserone et al., 1991, Acuna et al., 1998a). These findings suggest the possibility that different degrees of modification of the phytotoxin by the tuber tissues to yield less bioactive metabolities might be occurring. Such assumptions are consistent with the fact that many xenobiotics, such as herbicides, are converted after being introduced into plant tissue. The conversion, by oxidation, hydrolysis, or conjugation (usually to carbohydrates, glutathione, or amino acids), frequently leads to detoxification of the original bioactive compounds (Kaufman, 1976). It has also been found that many of these same transformations can be accomplished by microbial agents (Kaufman, 1976).

This paper describes a microbially induced conjugation of thaxtomin A (**1**) and thaxtomin B (**2**) to yield the *O*-glucosides **3** and **4**, respectively. It is perceived that these compounds may prove to be useful as reference markers in the search for similar plant-induced conjugates. Also described is the relative loss of bioactivity when the thaxtomin conjugates were applied to the surfaces of aseptically cultured potato minitubers.

MATERIALS AND METHODS

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

Equipment. Fast atom bombardment (FAB) mass spectra (MS) were obtained on a Finnigan MAT 8200 mass spectrometer. Nuclear magnet resonance (NMR) spectra were recorded for solutions in deuterated methanol on a Varian Unity 400 spectrometer operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shifts were referenced to the solvent resonances (δ_H, 3.30 ppm; and δ_C, 49.00 ppm).

Phytotoxin Conjugation and Isolation Procedures. The *Bacillus mycoides* isolate (HL-BM-1) was maintained and subcultured on a solid modified glucose medium containing 4 g of yeast extract (powder) Oxoid, 5 g of anhydrous D-glucose, 0.250 g of K_2HPO_4, 0.250 g of KH_2PO_4, 0.100 g of $M_gSO_4 \cdot H_2O$, 0.050 g of NaCl, and 0.005 g of $FeSO_4 \cdot 7H_2O$ and incubated at 25 °C.

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 ~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 autoclaved at 15.0 lb for 20 min. The oatmeal medium was then inoculated with 5 mL of a 3-day-old shake culture of *B. mycoides*. The cultures were then incubated at 26 °C on a rotary shaker. After 3 days, the cultures were incubated with thaxtomin A (King and Lawrence, 1996) (2.0 mg per flask), and the transformation was monitored by TLC of ethyl acetate extracts of 5 mL portions of the reaction mix. After 4 days, the reaction mixes in six separate flasks were individually extracted with one 150 mL portion of butan-2-ol. The butan-2-ol layer was dried over anhydrous sodium sulfate and the butan-2-ol removed in vacuo at 35 °C. The residue was taken up in methanol and fractionated on 0.5 mm silica gel 60A TLC plates with ethyl acetate/water/acetic acid/formic acid (10:1:1:1) to yield the crude thaxtomin A glucoside (R_f 0.42) as the only conversion product. The compound was rechromatographed on silica gel and purified further by reversed-phase TLC with acetone/water (3:2) to give pure thaxtomin A β-D-glucoside (**3**) (R_f 0.83). Thaxtomin B (King and Lawrence, 1996) was similarly conjugated and the glycoside purified to yield thaxtomin B β-D-glucoside (**4**) (R_f 0.82) on RP C_{18} TLC plates.

Phytotoxicity Assay. Aseptic minitubers of *Solanum tuberosum* L. cv. Green Mountain were produced and maintained in vitro by implanting sterile sections from dark-grown sprouts in White's amended medium containing 8% sucrose (King et al., 1991). Test materials were assayed for phytotoxic activity by appressing 4 mm antibiotic blank paper disks

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saturated with the material onto the surface of the minitubers. Lesions normally appeared within 24 h. After 48 h, the amount of tuber necrosis was visibly estimated for each of three tubers and the process was replicated three times.

Bacterium Identification. Whole cell fatty acid analysis of the bacterial contaminant by the MIDI microbial identification system (Sasser, 1990) characterized it as *Bacillus mycoides*.

Glucoside Hydrolysis. The glucoside (1 mg) was heated in a water bath (90 °C) with 5% HCl/EtOH (1 mL) for 20 min. The solution was diluted with water (5 mL) and the aglycon extracted with ethyl acetate for comparative TLC. The presence of glucose in the aqueous layer was confirmed by HPLC analysis (Coleman and King, 1984).

RESULTS AND DISCUSSION

During efforts to acquire larger amounts of the minor thaxtomins (for relative toxicity testing purposes) fractionation of the extracts from one particular oatmeal broth culture revealed that a substantial proportion of the major toxin thaxtomin A (**1**) had been converted into a much more polar compound. Reculturing of *S. scabies* colonies from the affected reaction flask indicated the presence of a bacterial contaminant, which was later characterized by fatty acid analysis as *B. mycoides*. That the bacterial contaminant *B. mycoides* had caused the transformation was confirmed when a small sample of thaxtomin A was added to an oatmeal broth culture of this bacterium.

After scaleup and purification by chromatographic means, correlated ^{1}H and ^{13}C NMR data for the modified toxin included adsorptions that were consistent with those previously assigned to thaxtomin A (**1**). However, its mass spectrum (FAB) indicated a major fragment at *m*/*z* 420 and a mass ion (M^+) of *m*/*z* 600, which is *m*/*z* 162 greater than that of thaxtomin A. This apparent conjugate also displayed ^{1}H and ^{13}C NMR spectral absorptions that could readily be ascribed to the presence of a carbohydrate group (Table 1).

That the carbohydrate group was glucose bonded in the β-configuration followed from its comparative ^{13}C NMR spectrum (Stothers, 1972), and use of the ^{1}H NMR anomeric proton at δ 4.11 as a starting point in the COSY spectrum allowed assignment of all the protons of the glucosyl ring (Table 1) (Coxon, 1972). Thereafter, heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments were used to confirm the chemical shift assignments and to determine the position of attachment of glucose to thaxtomin A. Observation of a correlation in the HMBC spectrum between C-14 of the aglycon and H′-1 of glucose indicated that the mode of linkage was through the C-14 hydroxyl group and not the more reactive C-20 phenolic, as might have been expected. This point of attachment was further corroborated when we were able to demonstrate that the *B. mycoides* isolate converted thaxtomin B (**2**) (which lacks the C-20 phenolic) to a glucoside conjugate (**4**) with ^{1}H NMR spectral properties comparable to those of the thaxtomin A glucoside (**3**) (Table 1). Further confirmation of the conjugate structures **3** and **4** was obtained by acid-catalyzed hydrolysis. Analysis of the reaction mix demonstrated the presence of glucose and the appropriate aglycon moieties.

Phytotoxic evaluation of the thaxtomin glucosides on aseptically produced potato minitubers indicated a substantial loss of bioactivity compared to the parent compounds. For example, at application rates of 0.1 and 0.5 μM the thaxtomin glucosides individually produced less than one-fifth of the tissue necrosis observed with the parent compounds. On the basis of these observations glucosylation of the thaxtomins can be viewed as a partial detoxification step.

If similar glucosylation processes are operative in the potato tuber and to different degrees depending on the particular cultivar, then the variable responses observed on treatment with thaxtomin A might be explained. Some indirect evidence for such a relationship has recently been presented by Acuna et al. (1998b); that is, a postulated thaxtomin A β-di-*O*-glucoside was generated when the protein extracts from potato stolons were incubated with UDP glucose and thaxtomin A. However, to our knowledge and from our own investigations, no direct evidence for the presence of thaxtomin A glucosides in either field or greenhouse scab-infected potatoes has yet been demonstrated. Nevertheless, these studies do indicate that manipulation of glucosylation processes (inherent or otherwise) in potato tubers might provide a venue for enhancement of resistance to the scab phytotoxins.

Table 1. NMR Spectral Assignments for Compounds 3 and 4

	3		4
carbon	^{13}C mult	^{1}H *J* (Hz)	^{1}H *J* (Hz)
2	132.79	6.86 s	6.69 s
3	110.47		
4	143.55		
5	119.30	7.84 dd, *J* = 8.0, 1.0	7.83 dd, *J* = 8.0, 1.0
6	120.91	7.19 t, *J* = 8.0	7.19 t, *J* = 8.0
7	118.43	7.70 dd, *J* = 8.0, 1.0	7.70 dd, *J* = 8.0, 1.0
8	119.93		
9	141.18		
10	49.94	1.71 dd, *J* = 14.2, 8.8	1.65 m
		2.69 dd, *J* = 14.0, 6.2	2.66 dd, *J* = 14.3, 6.0
11	64.32	4.01 dd, *J* = 8.6, 6.4	4.03 dd, *J* = 8.6, 6.1
13	166.52 s		
14	91.62		
16	168.41 s		
17	42.61	3.22 d, *J* = 13.6	3.22 d, *J* = 13.6
		3.49 d, *J* = 13.6	3.55 d, *J* = 13.6
18	136.18		
19	118.40	6.77 m	
20	161.90		
21	117.00	7.21−7.30 m	7.23−7.47 m
22	130.94		
23	121.39	6.77 m	
CH_3 (N-12)	29.33 q	3.08 s	3.11 s
CH_3 (N-15)	38.27 q	2.78 s	2.79 s
1′	98.9	4.11 d, *J* = 7.35	4.12 d, *J* = 7.4
2′	74.6	3.31[a]	3.31
3′	77.9	3.27	3.27
4′	70.8	3.27	3.27
5′	78.6	3.04 m	3.05 m
6′	62.2	3.52 dd, *J* = 12.0, 2.2	3.51 dd, *J* = 12.0, 2.2
		3.66 dd, *J* = 12.0, 2.1	3.68 dd, *J* = 12.0, 2.1

[a] Proton−proton coupling constants for 2′, 3′, and 4′ were obscured by the residual methanol peak.

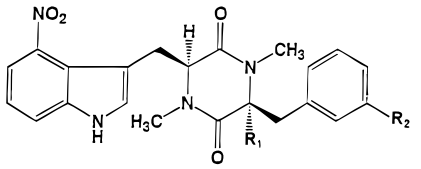

Figure 1. Structural formulas of compounds **1**−**4**: (**1**) R_1 = OH, R_2 = OH; (**2**) R_1 = OH, R_2 = H; (**3**) R_1 = Glu, R_2 = OH; (**4**) R_1 = Glu, R_2 = H.

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