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More chemistry of the thaxtomin phytotoxins

Russell R. King[a,*], C. Harold Lawrence, Jean Embleton[a], Larry A. Calhoun[b]

[a] *Agriculture and Agri-Food Canada, Potato Research Centre, PO Box 20280, Fredericton, New Brunswick, Canada E3B 4Z7*
[b] *Department of Chemistry, University of New Brunswick, Fredericton, New Brunswick, Canada E3B 6E2*

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Abstract

Chemical and biochemical studies indicated the possible involvement of *N*-acetyltryptophan and 4-nitrotryptophan as intermediates in biosynthesis of the thaxtomin phytotoxins. A search for other potential pathways indirectly resulted in the identification of three unusual thaxtomin analogues derived from the *o*-thaxtomin A isomer. Investigations to resolve the identity of a previously described thaxtomin A di-glucoside were not supportive of the proposed structure.

Keywords: Thaxtomin A; 4-Nitrotryptophans; *o*-Thaxtomin A; Auto-oxidation; *Streptomyces scabies*; *Thaxtomin A glucosides*

1. Introduction

Investigations of phytotoxins generated in vitro by *Streptomyces* genotypes predominantly associated with the common scab of potato disease have resulted in the isolation and characterization of a series of unique 4-nitroindol-3-yl containing 2,5-diketopiperazines (Loria et al., 1997 and references therein). These phytotoxins, named thaxtomins, can cause plant cell necrosis at nanomolar concentrations. Injury symptoms in most instances are similar to those caused by known cellulose biosynthetic inhibitors (King et al., 2001). To date, a total of 11 related compounds (Fig. 1) have been isolated and characterized (King et al., 1989, 1992, 1994; King and Lawrence, 1996). Thaxtomin A (**1**) is the most prominent member of this group and its proposed structure has now been confirmed by X-ray analysis (Wagner, 2000). Inclusion of a 4-nitroindole moiety makes the thaxtomins unique amongst microbial generated metabolites. This feature is also essential for phytotoxic activity. As a consequence, the sequence and methodology of nitration are items of particular interest (King and Lawrence 1995; King et al., 1998; Wach et al., 2002) and some progress has been forthcoming. For instance, the identification of *N*-acetyl-4-nitrotryptophan (**12**) and *N*-methyl-4-nitrotryptophan (**13**) residues (Fig. 2) in the phytotoxin production medium (King and Lawrence, 1995) imply a pathway in which nitration of the indole moiety precedes formation of the diketopiperazine ring. These findings also indicate the probable involvement of *N*-methyl-4-nitrotryptophan as an immediate precursor in the formation of thaxtomin C (**3**) a fundamental member of the thaxtomin group. In an attempt to further unravel the intricacies of the nitration sequence we undertook exploration of the role that *N*-acetyl-4-nitrotryptophan (**12**) might play in the process. As a result of these investigations we now report the identification of two more candidates for consideration as potential thaxtomin intermediates. Results from an associated study which led to the isolation and characterization of three unusual transformation products derived from the *o*-thaxtomin A analogue (**2**) are also presented.

In another development of interest, Acuna et al. (2001), reported the isolation of a thaxtomin A di-glucoside produced in vitro by *Streptomyces scabies*. This compound was subsequently utilized as a standard by which to measure the apparent glucosylation of thaxtomin A in potato tubers. To our knowledge however, substantial differences between the TLC mobility of the purported thaxtomin A di-glucoside and other known diketopiperazine glucosides (Park et al., 1994; King et al., 2000) would clearly preclude such a close relationship (see text for details). In contrast, the TLC retention

* Corresponding author. Tel.: +1-506-452-4884; fax: +1-506-452-3316.
E-mail address: kingr@agr.gc.ca (R.R. King).

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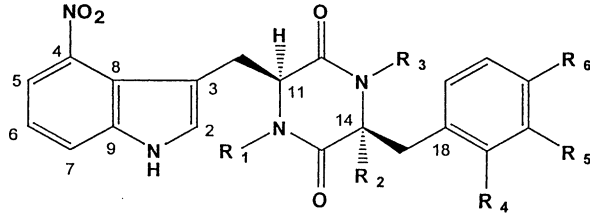
time of the supposed di-glucoside appears virtually identical to that of the previously described hydroxythaxtomin-A analogue **8** (King and Lawrence, 1996). These and other inconsistencies associated with assignment of the di-glucoside structure prompted us to undertake a comparable investigation of thaxtomin A derivatives generated in vitro by the appropriate *Streptomyces scabies* strain. As part of this report we contrast our findings with those of the Acuna et al. (2001) study and further elaborate on disparities found therein.

2. Results and discussion

The manner by which a nitro group is incorporated into the indole part of the thaxtomin molecule has not yet been established. The lack of success experienced in attempts to induce thaxtomin production in a synthetic medium (Babcock et al., 1993) is undoubtedly one of the impediments to such studies. It has also been demonstrated that thaxtomin A production is repressed in a natural medium by the addition of tryptophan (Babcock et al., 1993; Lauzier et al., 2002). As a starting point in examining the process by non-isotopic means we decided to first explore the relative roles of *N*-acetyl-4-nitrotryptophan (**12**) and *N*-methyl-4-nitrotryptophan (**13**) (Fig. 2), two compounds consistently detected in the water soluble exudates associated with the in vitro production of thaxtomin A (King and Lawrence, 1995). It is quite apparent that condensation of *N*-methyl-4-nitrotryptophan (**13**) with phenylalanine would produce 12-*N*-methylcyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (**3**) a member of the thaxtomins that is a logical precursor for the synthesis of all but one of the other thaxtomins presently characterized. In the case of *N*-acetyl-4-nitrotryptophan (**12**), its role is less immediately obvious, it could represent an intermediate from another pathway or a precursor to the *N*-methyl analogue (**13**). As evidence for the latter role we observed in our thaxtomin A production studies (King and Lawrence, 1995) that the ratio of *N*-acetyl-4-nitro-tryptophan (**12**) to *N*-methyl-4-nitrotryptophan (**13**) usually decreased over time. We next determined that when added to isolates of *Streptomyces scabies* cultured in a minimal medium substantial portions of *N*-acetyl-4-nitrotryptophan (**12**) were transformed into 4-nitrotryptophan (**14**), a feasible precursor to both cyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (**11**) and *N*-methyl-4-nitrotryptophan (**13**). On the basis of these findings we undertook a detailed TLC–HPLC investigation of the exudates associated with thaxtomin A production in a search for any potential precursors to *N*-acetyl-4-nitrotryptophan (**12**) or *N*-methyl-4-nitrotryptophan (**13**). As initial search guides we concentrated on substances that demonstrated retention times in the near vicinity of *N*-acetyl-tryptophan (**15**) and *N*-methyltryptophan (**16**) on silica gel TLC plates (Table 1). It was subsequently determined that trace amounts of both 4-nitrotryptophan (**14**) and *N*-acetyl-tryptophan (**15**) could

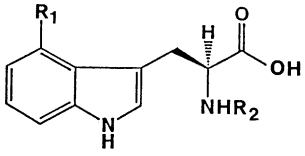


Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	Me	OH	Me	H	OH	H
2	Me	OH	Me	OH	H	H
3	Me	H	H	H	H	H
4	Me	OH	Me	H	H	H
5	Me	H	Me	H	H	H
6	Me	OH	H	H	H	H
7	Me	OH	Me	H	H	OH
8	Me	OH	Me	H	OH	OH
9	Me	OH	H	H	OH	H
10	H	OH	Me	H	OH	H
11	H	H	H	H	H	H

Fig. 1. Structural formulas of the thaxtomins.

Table 1
Summary of chromatography data for compounds **12–16**

Compd	TLC R_f values		HPLC R_t
	Silica gel	RP-C_{18}	(min)
12	0.74	0.94	27.4
13	0.21	0.81	22.4
14	0.22	0.76	28.3
15	0.80	0.94	16.4
16	0.25	0.85	9.3



12	$R_1 = NO_2$	R_2 = Ac
13	$R_1 = NO_2$	R_2 = Me
14	$R_1 = NO_2$	$R_2 = H$
15	$R_1 = H$	R_2 = Ac
16	$R_1 = H$	R_2 = Me
17	$R_1 = NH_2$	R_2 = Ac

Fig. 2. Structural formulas of compounds **12–17**.

consistently be detected (by HPLC-UV) in some TLC fractions. On the assumption that *N*-acetyl-4-aminotryptophan (**17**) might be an intermediate in the biosynthesis of *N*-acetyl-4-nitrotryptophan (**12**) we proceeded to examine the water soluble exudates associated with thaxtomin A production for the presence of any compounds exhibiting UV spectra comparable to 4-aminoindole. However, these investigations ultimately failed to provide any solid evidence for the existence of 4-aminoindole containing compounds. From these results we are left to speculate whether the nitration process is (i) a concerted reaction proceeding through relatively unstable tryptophan intermediates, (ii) occurs by direct nitration of tryptophan or (iii) takes place prior to the tryptophan stage.

With regard to the latter option, a previous study (King et al., 1998) had provided some evidence for the possible introduction of a nitro group at the anthranilic acid stage of tryptophan biosynthesis but the results were indeterminate. With this route still a possibility we next focussed our efforts on the accumulation and characterization of another non-phytotoxic trace metabolite frequently detected in the ethyl acetate soluble exudates associated with in vitro production of the thaxtomins. Subsequent NMR studies indicated that the metabolite contained a 4-nitroindole moiety but its ^{1}H NMR spectrum (Table 2) did not display the usual phenyl group and associated methylene protons. Concurrently its ^{13}C NMR spectrum indicated the probable presence of a C-13, C-14 quinone system (Table 2). These data taken in conjunction with a molecular formula deduced as $C_{15}H_{14}N_4O_5$ from MS data suggested the structure **18** for the compound (Fig. 3). A rationale for the production of this compound was not readily apparent. That it might represent an intermediate of another biosynthetic route to thaxtomin A seemed a definite possibility. Endeavours to trace the origins of this new analogue however, eventually revealed that it was probably being generated through degradation of the *o*-thaxtomin A isomer (**2**). For example, detailed examination of the *o*-thaxtomin A band from TLC indicated that small traces of two other compounds were always inherent. Attempts to purify these new compounds were somewhat complicated by their instability. The more predominant of the two demonstrated a tendency to partially reconvert to *o*-thaxtomin A (**2**). This reconversion process could best be accounted for if isomerization of the C-14 hydroxyl group was taking place to yield the C-14 epimer **19**. This explanation was considerably strengthened when MS and NMR spectroscopy data (Table 3) proved compatible with such a structural designation.

Table 2
^{1}H and ^{13}C NMR data for compound **18** in acetone-d_6

Pos	δ_H mult (*J*, Hz)	δ_C[a] mult[b]	HMBC[c]
1	11.1 *br s*		
2	7.3 *d* (2.7)	131.0 *d*	3
3		106.2 *s*	
4		142.4 *s*	
5	7.84 *m*	117.7 *d*[d]	4
6	7.28 *t* (8.0)	120.5 *d*	4, 9
7	7.84 *m*	118.5 *d*[d]	
8		118.3 *s*	
9		139.2 *s*	
10	3.81 *ddd* (14.9, 4.1, 0.5)	30.4 *t*	
	3.54 *ddd* (14.9, 5.0, 0.4)		2, 3, 8, 11
11	4.61 *dd* (5.0, 4.1)	64.3 *d*	13
12N-Me	3.13 *s*	32.0 *q*	11, 13
13		151.9 *s*	
14		155.9 *s*	
15N-Me	2.5 *s*	25.3 *q*	14, 16
16		168.0 *s*	

[a] Data derived from HMQC and HMBC spectra.
[b] Multiplicity inferred from ^{1}H and HMQC spectra.
[c] Optimized for $J=8$ Hz. Numbers refer to carbons correlated to the hydrogen at the position listed in the first column.
[d] Assignments may be interchanged due to overlap of H5 and H7.

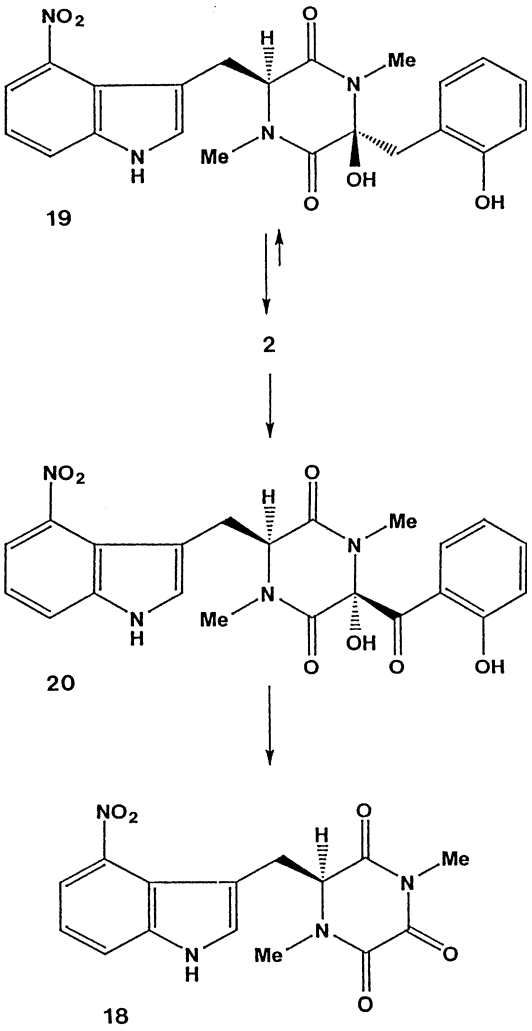


Fig. 3. Structural formulas of transformation products derived from *o*-thaxtomin A.

The ^{1}H NMR spectrum was especially revealing since previous studies of synthetic thaxtomin epimers had highlighted significant differences in protons located at the C-10 position (King et al., 1992). These same differences were also quite apparent in the epimers **2** and **19**. For example, in their respective ^{1}H NMR spectra epimer **2** displayed H-10 proton signals at δ 1.96 and 2.83 (King et al., 1992) while epimer **19** had related absorptions at δ 3.32 and 3.52. These differences can be ascribed to conformational iniquities between the two compounds. The isomerization process is undoubtedly induced in some manner by the proximity of the *ortho* phenol group since a similar type of conversion has not been observed to occur in any other members of the thaxtomin group. Characterization of the other new compound was somewhat more complex. It was observed to partially decompose on TLC to yield the afore described diketopiperazine **18**. Its ^{1}H NMR data (Table 4) indicated a lack of C-17 methylene protons and high resolution MS studies confirmed the addition of an oxygen atom. These data appear consistent only with formation of the C-17 keto analogue **20** and is compatible with the observed presence of three carbonyl related absorptions in its ^{13}C NMR spectrum (Table 4). Generation of this compound from *o*-thaxtomin A (**2**) would appear to be the result of an auto-oxidation process possibly initiated and encouraged by the proximity of the *ortho* phenol group. A further oxidation step could result in its observed decomposition to compound **18**. As expected from the results of related studies, both the *o*-thaxtomin A isomer (**19**) and compound **18** proved essentially devoid of any bio-activity on mini-tubers (King et al., 2000). Compound **20**, though not completely inactive, exhibited effects much reduced in comparison to the parent *o*-thaxtomin A (**2**).

In summation, some further evidence regarding a potential route for introduction of a nitro group into the indole moiety of the thaxtomins has been elaborated. As a related result of these studies, the structures of several interesting new thaxtomin analogues have also been established.

In an attempt to confirm or disprove generation of a thaxtomin A di-glucoside, *Streptomyces scabies* strain 87-22 provided by Dr. Rosemary Loria, Cornell University, Ithaca, NY was grown on an oatmeal medium and the secondary metabolites extracted and purified as described by Acuna et al. (2001). The major metabolite proved identical to a standard sample of thaxtomin A but as in previous studies with other *Streptomyces scabies* isolates we could not detect any trace of thaxtomin A conjugates (King and Lawrence, unpublished results). However, a minor metabolite which displayed a reten-

Table 3
^{1}H and ^{13}C NMR data for compound **19** in MeOH-d_4

Pos	δ_H mult (J, Hz)	δ_C mult[b]	HMBC[c]
1	[a]		
2	7.13 *s*	129.23 *d*	3, 8, 9, 10
3		108.99 *s*	
4		144.38 *s*	
5	7.71 *dd* (7.9, 1.0)	118.15 *d*	4, 7, 8
6	7.17 *t* (8.0)	121.06 *d*	4, 9
7	7.66 *dd* (8.0, 1.0)	118.55 *d*	5, 8
8		120.20 *s*	
9		140.60 *s*	
10	3.52 *ddd* (16.0, 3.8, 0.8)	29.70 *t*	2, 3, 8, 11, 16
	3.25 *ddd* (15.9, 4.6, 0.8)		
11	3.12 *br t* (4.3)	62.31 *d*	3, 12N-Me, 13, 16
12N-Me	2.84 *s*	28.34 *q*	11, 13
13		169.23 *s*	
14		87.18 *s*	
15N-Me	2.78 *s*	32.73 *q*	14, 16
16		167.24 *s*	
17	3.32 *d* (13.5)	39.01 *t*	13, 14, 18, 19, 23
	3.07 *d* (13.6)		
18		121.40 *s*	
19		157.50 *s*	
20	6.70 *m*	116.05 *d*	22
21	7.06 *m*	129.88 *d*	19, 23
22	6.68 *m*	120.13 *d*	20
23	6.86 *dd* (7.9, 1.8)	132.39 *d*	17, 19, 21

[a] Not observed.
[b] Multiplicity inferred from ^{1}H and HMQC spectra.
[c] Optimized for J=8 Hz. Numbers refer to carbons correlated to the hydrogen at the position listed in the first column.

Table 4
^{1}H and ^{13}C NMR data for compound **20** in acetone-d_6[a,b]

Pos	δ_H mult (J, Hz)	δ_c^{γ} mult	HMBC[c]
1	11.02 *br s*		
2	7.49 *d* (2.7)	131.77 *d*	3, 8, 9
3		110.0 *s*	
4		143.54 *s*	
5	7.92 *dd* (7.9, 1.0)	118.31 *d*	7
6	7.30 t (8.0)	121.12 *d*	4, 9
7	7.88 *dd* (8.1, 1.0)	119.25 *d*	5, 8
8		120.0 *s*	
9		140.6 *s*	
10	3.74 *ddd* (14.2, 7.3, 0.7)	33.25 *t*	2, 3, 8, 11, 16
	3.64 *ddd* (14.2, 7.5, 0.7)		2, 3, 8, 11, 16
11	4.39 *t* (7.4)	64.69 *d*	11, 13, 16
12N-Me	2.78 s	33.67 *q*	11, 13
13		164.8 *s*	
14		87.18 *s*	
15N-Me	2.59 *s*	28.6 *q*	14, 16
16		168.0 *s*	
17		197.7 *s*	
18		117.2 *s*	
19		163.9 *s*	
20	6.99 *br d* (8.3)	119.3 *d*	18, 22
21	7.55 *br t* (8.3)	138.26 *d*	19, 23
22	6.85 *br t* (7.7)	120.1 *d*	18, 20
23	7.83 *dd* (8.2, 1.5)	130.77 *d*	17, 19, 21

[a] Chemical shifts listed to one decimal place are derived from HMQC and HMBC spectra.
[b] Multiplicity inferred from ^{1}H and HMQC spectra.
[c] Optimized for J=8 Hz. Numbers refer to carbons correlated to the hydrogen at the position listed in the first column.

tion time similar to the purported thaxtomin A di-glucoside in the appropriate TLC systems was recovered. This minor metabolite though, co-migrated with an authentic sample of hydroxy-thaxtomin A (**8**) (King and Lawrence, 1996) in the recommended TLC systems (Acuna et al., 2001) and had similar spectral properties. That the identity of this metabolite was incorrectly assigned by the Acuna group is further evident when its TLC retention time is compared with other diketopiperazine glucosides. For example, when subjected to silica TLC plate chromatography in a chloroform–methanol (9:1) solvent system the supposed di-glucoside displayed a retention time of 0.21 (Acuna et al., 2001). This contrasts poorly with a diketopiperazine mono-glucoside such as maculosin-glucoside (Park et al., 1994) which had a recorded retention time of only 0.09 in a much more polar solvent system i.e., chloroform–methanol–acetic acid (7:1:0.5). More telling was our finding that even a mono-glucoside of thaxtomin A (King et al., 2000) would not migrate from the baseline on a silica gel TLC plate run in chloroform–methanol (9:1). Aside from chromatographic inconsistencies there were other features that indicated the Acuna et al. study had a chemical characterization problem. For example, the most prominent NMR spectral data presented to support their structural elucidation was the detection of supposed glucosyl anomeric protons at δ 5.3–5.4. However, in our characterization studies of the minor metabolites this is precisely where we frequently detected signals associated with co-extracted material. The co-extractives could be removed by further TLC procedures. These latter observations suggest that the Acuna et al. study may also have had a sample purity problem which could compromise both phytotoxicity and hydrolysis results. Inexplicably, there was no mention in their report of any efforts to detect thaxtomin A residues in the hydrolysate from the purported thaxtomin A di-glucoside. If our contentions regarding the identity and purity of their quantifying standard are correct then any results obtained utilizing that standard are unreliable. We therefore suggest that the whole concept of thaxtomin A glucosylation as a means of detoxification in potato tubers is greatly in need of a serious re-examination.

3. Experimental

3.1. General

All solvents used were HPLC grade. *N*-Acetyl-L-tryptophan, *N*-methyl-L-tryptophan and 4-aminoindole were purchased from Sigma-Aldrich Canada Ltd.

Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} plates and Whatman $KC_{18}F$ plates. High-performance liquid chromatography (HPLC) studies utilized a Hewlett-Packard HPLC 1100 system and Supercosil LC-18, 15 cm×4.6 mm×5 μm column eluted with water–acetonitrile–acetic acid (86:13:1) at a flow rate of 1.0 ml/min monitored with a Shimadzu SPD-M6A diode array UV–vis detector.

Chemical ionization (CI) and electrospray (ES) mass spectra (MS) were obtained on a Finnigan MAT 312 mass spectrometer and a Micromass LCT mass spectrometer, respectively. Nuclear magnetic resonance (NMR) spectra were recorded for solutions in deuterated methanol (unless otherwise indicated) on a Varian Unity 400 spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C. Chemical shifts were referenced to the solvent resonances (δ_H 3.30 ppm; and δ_C 49.00 ppm).

3.2. Isolation of intermediates

Reaction mixes from oatmeal broth media inoculated with various *Streptomyces scabies* isolates (King et al., 1992) were extracted with equivalent portions of butan-2-ol. The butan-2-ol layers were dried over anhydrous sodium sulfate and the butan-2-ol removed in vacuo at 35 °C. The residues were taken up in methanol and fractionated on 0.5 mm silica gel 60 A TLC plates with ethyl acetate–water–acetic acid–formic acid (20:1:1:1). Selected fractions were subjected to further clean-up by reversed-phase TLC with acetone–water (3:2) prior to HPLC analysis.

3.3. 4-Nitrotryptophan (*14*)

Conversion of *N*-acetyl-4-nitrotryptophan (**12**) to 4-nitrotryptophan (**14**) was achieved by adding *N*-acetyl-4-nitrotryptophan (**12**) (2 mg) to 100 ml portions of 3 day old shake cultures of the test organisms in a *Streptomyces* growth medium (SGM) (King et al., 1994). After 4–5 days the reaction mix was extracted with an equivalent portion of butan-2-ol and the residue fractioned on 0.25 mm silica gel 60A TLC plates in an ethyl acetate–water–acetic acid–formic acid (20:1:1:1) solvent system to give 4-nitrotryptophan (**14**) as a yellowish solid. MS (CI) *m*/*z* 249 [M^+, $C_{11}H_{11}N_3O_4$]. ^1H NMR (DMSO-d_6): δ 11.79 (NH, *br s*, H-1), 7.78 (1H, *dd*, J=7.9, 0.9 Hz, H-5), 7.76 (1H, *dd*, J=7.9, 0.9 Hz, H-7), 7.57 (1H, *m*, H-2), 7.23 (1H, *t*, J=7.9 Hz, H-6), 3.42 (1H, *dd*, J=15.3, 4.5 Hz, H-10a), 3.32 (1H, *dd*, J=9.7, 4.5 Hz, H-11), 2.89 (1H, *dd*, J=15.3, 9.7 Hz, H-10b).

3.4. Isolation of metabolites

An accumulated quantity of crude *o*-thaxtomin A (**2**)(69 mg) was fractionated on 0.5 mm silica gel 60A TLC plates with a solvent system of chloroform–methanol (9:1). In addition to pure *o*-thaxtomin A (**2**), this procedure also yielded fractions containing small quantities (2–4 mg) of compounds **18**, **19** and **20**. These latter compounds were subsequently purified further (as

described above) to furnish pure compound 18 (R_f 0.47) as a yellow solid with MS (CI) m/z 330.1 [M^+, calc. for $C_{15}H_{14}N_4O_5$ 330.0965], compound **19** (R_f 0.31) as a yellow solid with MS (ES) m/z 438.1538 [M^+, calc. for $C_{22}H_{22}N_40_6$ 438.1539] and compound **20** (R_f 0.62) as a yellow solid with m/z 452.1336 [M^+, calc. for $C_{22}H_{20}N_4O_7$ 452.1332. For ^1H and ^{13}C NMR spectral data see Tables 2–4.

3.5. Metabolite production

Streptomyces scabies strain 87-22 obtained from the collection of Dr. Rosemary Loria, Cornell University, NY was cultured on oatmeal broth and the metabolites extracted and purified on TLC as described by Acuna et al. (2001). Relevant metabolites were identified by comparison of TLC and HPLC retention times, UV and NMR spectra of the appropriate standards (King and Lawrence, 1996). The metabolite with a retention time corresponding to that of the purported thaxtomin A diglucoside was referenced to an authentic sample of hydroxy-thaxtomin A (**8**) in the four solvent systems utilized by the Acuna group for a purity check.

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