Chemistry of Phytotoxins Associated with *Streptomyces scabies*, the Causal Organism of Potato Common Scab

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Extensive chromatography of *Streptomyces scabies* infected potato tissue yielded five compounds which on aseptically cultured minitubers individually reproduced symptoms typical of the common scab disease. Spectroscopic examination of the minor components elicited sufficient information to identify them as related analogues of the major constituents thaxtomin A and thaxtomin B, which had been previously characterized as unique 4-nitroindol-3-yl-containing 2,5-dioxopiperazines. Subsequently, a number of synthetic variants were prepared to further understanding of the structural requirements for phytoactivity in this class of compounds.

Due to its detrimental effect on appearance, grade, and quality, common scab of potato [caused by the bacterium *Streptomyces scabies* (Thaxt.) Lambert and Loria] is considered a disease of major economic importance in many potato-producing areas of the world. Attempts at this institution to elucidate the host–parasite interaction were successful in demonstrating that phytotoxins produced extracellularly by pathogenic isolates would induce the development of scablike lesions on aseptically cultured potato minitubers (Lawrence et al., 1990; King et al., 1991). The major phytotoxins, provisionally named thaxtomin A (1) and thaxtomin B (4), were successfully isolated and characterized by a combination of spectral methods and partial syntheses as 4-nitroindol-3-yl-containing 2,5-dioxopiperazines. In this connection we have already published a preliminary paper (King et al., 1989). We now present a full report on the chemistry of thaxtomins A and B and of three previously unreported analogues. Also outlined are the results of structure–activity relationship studies involving 5-nitroindoyl, 6-nitroindolyl, 7-nitroindoyl, and desphenylalanine analogues.

MATERIALS AND METHODS

Chemicals. Samples of *o*-, *m*-, and *p*-phenylacetic acid and all standard amino acid derivatives were purchased from Sigma Chemical Co., St. Louis, MO. Nitrotryptophan derivatives were prepared as outlined by Calhoun and King (1992).

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. High-performance liquid chromatography (HPLC) utilized a Perkin-Elmer Series 4 liquid chromatograph and a Supelco RP-18 column (5-μm particle size, 4.6 mm × 15 cm) eluted with water/acetonitrile 75:25 (v/v) at a flow rate of 1 mL/min and monitored at 380 nm with a UV detector.

Equipment. Melting points are uncorrected and were determined on a Kofler hotstage microscope. Infrared (IR) spectra were determined by using a Perkin-Elmer 467 grating infrared spectrophotometer. 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 ^{13}C. Chemical shifts were measured downfield from the signal of internal tetramethylsilane.

Phytotoxin Production and Isolation Procedures. Pathogenic isolates of *S. scabies* (King et al., 1991) and *Streptomyces ipomoea* 7857 were 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 $MgSO_4 \cdot H_2O$, 0.050 g of NaCl, and 0.005 g of $FeSO_4 \cdot 7H_2O$ and incubated at 25 °C. Field-grown tubers (cv. Green Mountain and Russet Burbank) were washed, peeled, surface sterilized with Javex (10%), and cut into thin slices (0.25-cm thickness). The tuber slices (ca. 100 g/batch) were transferred to sterile Petri dishes and inoculated with a 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-layer chromatography on 0.2-mm RP-C_{18} with acetone/water (3:2). 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 0.25-mm silica gel 60 TLC plates with chloroform/methanol (9:1).

Synthetic Procedures. Sterically pure diketopiperazines cyclo-(L-tryptophyl-L-phenylalanyl), cyclo-(L-4-nitrotryptophyl-L-phenylalanyl), cyclo-(L-5-nitrotryptophyl-L-phenylalanyl), cyclo-(L-6-nitrotryptophyl-L-phenylalanyl), cyclo-(L-7-nitrotryptophyl-L-phenylalanyl), cyclo-(L-4-nitrotryptophyl-L-leucyl) and their D,L isomers were prepared according to established procedures (Nitecki et al., 1968) from the appropriate amino acid precursors. Subsequently, it was discovered that the 4-nitro analogues could also be obtained by nitration with nitric acid plus acetic acid (Hester, 1964) of the appropriate 2,5-dioxopiperazine precursors. N-Methylation procedures utilized sodium hydride and methyl iodide in dimethylformamide as detailed by Brimacombe et al. (1966).

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Table I. Summary of Isolation Data for Phytotoxins

compd	MW/formula	R_f values: RP C_{18}	R_f values: silica gel	yield, mg/100 g
1	438/$C_{22}H_{22}N_4O_6$	0.76	0.27	$<10^{-1}$
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	$<10^{-4}$
4	422/$C_{22}H_{22}N_4O_5$	0.66	0.41	$<10^{-3}$
5	406/$C_{22}H_{22}N_4O_4$	0.62	0.45	$<10^{-3}$

RESULTS AND DISCUSSION

Fractionation by reversed-phase TLC of the acetone-soluble extracts from *S. scabies* infected potato tissue yielded five components (one major, one intermediate, and three minor) that when applied to aseptically cultured minitubers individually reproduced symptoms typical of the common scab disease. The bioactive components were then subjected to further cleanup by silica gel TLC. This procedure eventually furnished chromatographically (HPTLC and HPLC) homogeneous entities that were yellow and exhibited relatively similar UV, IR, and MS characteristics. Table I summarizes isolation and molecular weight data.

Compound 1 (previously designated thaxtomin A; King et al., 1989) crystallized from methanol/acetone as pale orange rosettes: mp 230 °C (decomp); M^+ 438.1708; λ_{max} (EtOH) 398 (ϵ 4050), 343 (3220), 279 (5830), 249 (15070) and 220 (27700) nm; V_{max} (Nujol) 3200–3380, 1650, 1600, and 1510 cm^{-1}. The molecular formula of thaxtomin A was deduced as $C_{22}H_{22}N_4O_6$ from high-resolution FAB mass spectra and DEPT ^{13}C NMR experiments [6 sp^3 (1s, 1d, 2t and 2q) and 16 sp^2 (8s and 8d) hybridized carbon signals observed]. ^1H NMR indicated the presence of 19 unexchangeable protons, 8 of which were of an aromatic or alkenic nature. An analysis of the ^1H and COSY spectra revealed the presence of seven distinct spin systems: two *N*-methyl singlets, one isolated AB quartet characteristic of a methylene group adjacent to two fully substituted centers, an ABX system with the AB quartet consistent with a geminal proton pair in a highly anisotropic environment further coupled to an adjacent methine, an AMX system typical of three ortho-coupled aromatic protons, an AMPX system reminiscent of an ortho- or meta-substituted benzene ring, and finally a singlet aromatic proton.

The presence of nitroindol-3-yl and benzyloxy groups in thaxtomin A (1) was first suggested from high-resolution mass spectral analysis (EI) of intense fragments at *m/z* 175 ($C_9H_7N_2O_2$), 162 ($C_8H_6N_2O_2$), 116 (C_8H_6N), 107 (C_7H_7O), and 89 (C_7H_5). Subsequent analysis of the ^1H homonuclear shift correlated (COSY) spectra indicated substitution in the C-4 position of the indol-3-yl nucleus and at the meta position of the benzyloxy moiety. Comparative NMR studies with related synthetic compounds (i.e., *N*-methyl-4-nitrotryptophan and *m*-hydroxyphenylacetic acid) then facilitated complete carbon and proton assignments for the aromatic positions (Tables II and IV). On the basis of the unassigned ^1H and ^{13}C NMR signals, the remaining subunit, i.e., $C_6H_8N_2O_3$, had to accommodate two *N*-methyl groups, two carbonyl moieties, an sp^3 carbon atom compatible with the presence of a tertiary hydroxyl group, plus a one-proton quartet which was assigned to the C-11 position on the basis of an observed allylic coupling between the B proton of the ABX spin system and the 2-H proton of the 4-nitroindol-3-yl moiety. This information together with the IR data which displayed disubstituted amide bands at 1650 cm^{-1} was rationalized as indicative of an *N,N*-dimethyl-2,5-dioxo-3-hydroxypiperazine unit coupling the 4-nitroindol-3-yl and *m*-hydroxybenzyl groups. For the purposes of structural confirmation and determination of relative stereochemistry (at C-11 and C-14) cyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (6) and cyclo-(D-4-nitrotryptophyl-L-phenylalanyl) were prepared. Significantly, only the former compound exhibited thaxtomin A-like activity.

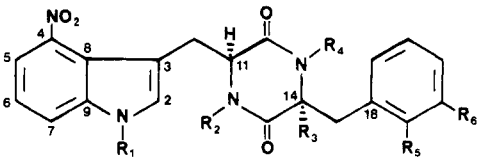

Figure 1. Structural formulas of compounds 1–7.

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	OH	H
3	H	Me	H	H	H	H
4	H	Me	OH	Me	H	H
5	H	Me	H	Me	H	H
6	H	H	H	H	H	H
7	Me	Me	H	Me	H	H

Mass spectrometry revealed that compound 2 was also of composition $C_{22}H_{22}N_4O_6$ and had a similar fragmentation pattern to that of thaxtomin A (1). Subsequent assignment of the structure for compound 2 as the *o*-benzyloxy isomer of thaxtomin A was based on ^1H COSY spectra and comparative studies with a synthetic sample of *o*-hydroxyphenylacetic acid and thaxtomin A (1). Compound 2 also crystallized as pale orange rosettes from acetone/methanol and had mp 228 °C (decomp).

The amount of compound 3 originally acquired from *S. scabies* infected potato tissue was insufficient to obtain a satisfactory ^1H NMR spectrum. However, in a fortunate coincidence, related investigations examining the host–parasite relationship of *S. ipomoea* (the organism responsible for common scab of sweet potato) yielded (as the predominant phytotoxin) moderate quantities of a substance that proved to be identical (MS, TLC, and UV) to compound 3. A comparative analysis of its ^1H NMR spectral data (Table III) established the structure of compound 3 as 12-*N*-methylcyclo-(L-4-nitrotryptophyl-L-phenylalanyl). Assignment of the *N*-methyl substituent was determined on the basis of an observed coupling between H-14 and an adjacent amide proton. The structural elucidation of compound 3 was also 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) (7).

Compound 4 (thaxtomin B) crystallized from methanol/acetone as pale orange needles with mp 238 °C (decomp) and had M^+ 422.1753, which corresponded to a molecular formula of $C_{22}H_{22}N_4O_5$. The structure of thaxtomin B (4) as a C-20 deoxy analogue of thaxtomin A (1) was readily apparent from comparative analysis of its ^1H NMR and ^{13}C NMR spectra (see Tables II and IV) and had been identified in our earlier work (King et al., 1989).

Compound 5 was originally characterized as a C-14 deoxy analogue of thaxtomin B (4) from its mass spectral data and ^1H NMR decoupling experiments (see Table III). Subsequent methylation of its indole nitrogen with MeI/NaH also confirmed the structural assignment by providing a derivative that was directly comparable to a synthetic sample of 1,12,15-*N,N,N*-trimethylcyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (7).

From assessment of the structural determinations it is apparent that 12-*N*-methylcyclo-(L-4-nitrotryptophyl-L-phenylalanyl) (3) constitutes the most fundamental member of the thaxtomin group yet identified. Accordingly,

Table II. ^{1}H NMR Spectral Assignments for Compounds 1, 2, and 4

hydrogen	1	2	4
2	6.95 s	6.92 s	6.84 s
5	7.84 dd, J^a = 7.9, 1.0	7.84 dd, J = 7.8, 1.0	7.84 dd, J = 7.9, 1.0
6	7.19 t, J = 8.0	7.20 t, J = 8.0	7.20 t, J = 8.0
7	7.68 dd, J = 8.1, 1.0	7.71 dd, J = 8.1, 1.0	7.70 dd, J = 8.1, 1.0
10	1.62 dd, J = 14.2, 8.9	1.96 dd, J = 14.1, 9.0	1.64 dd, J = 14.4, 8.7
	2.60 ddd, J = 14.1, 6.2, 0.5	2.83 ddd, J = 14.2, 6.0, 0.6	2.63 dd, J = 14.3, 6.0, 6.0
11	3.86 dd, J = 8.9, 6.3	3.90 dd, J = 8.9, 6.0	3.89 dd, J = 8.7, 6.0
17	3.11 d, J = 13.4	3.13 d, J = 13.5	3.14 d, J = 13.6
	3.32 d, J = 13.5	3.44 d, J = 13.4	3.29 d, J = 13.6
19	6.71 m		7.21–7.44 m
20		6.85 m	
21	6.71 m	7.12 m	
22	7.23 t, J = 8.1	6.85 m	
23	6.71 m	7.12 m	
CH_3(N-12)	3.03 s	2.98 s	3.01 s
CH_3(N-15)	2.81 s	2.86 s	2.84 s

[a] J values in hertz.

Table III. ^{1}H NMR Spectral Assignments for Compounds 3 and 5–7

hydrogen	3[a]	5	6	7
2	7.21 s	7.09 s	7.10 s	7.00 s
5	7.95 dd, J^b = 7.9, 1.0	7.85 dd, J = 7.9, 1.0	7.84 dd, J = 7.9, 0.9	7.86 dd, J = 7.8, 0.9
6	7.26 m	7.23 dd, J = 8.0	7.21 t, J = 8.0	7.29 t, J = 8.1
7	7.65 dd, J = 8.1, 1.0	7.73 dd, J = 8.1, 1.0	7.72 dd, J = 8.1, 0.9	7.76 dd, J = 8.2, 0.9
10	2.93 dd, J = 14.5, 7.0	2.41 dd, J = 14.5, 7.5	2.17 dd, J = 14.0, 8.3	2.2 ddd, J = 14.4, 8.0, 0.4
	3.61 dd, J = 14.6, 4.6	2.97 m	3.20 dd, J = 14.4, 4.4	2.96 ddd, J = 14.5, 5.4, 0.6
11	4.12 dd, J = 7.0, 4.5	3.97 dd, J = 7.6, 5.8	4.01 ddd, J = 8.0, 4.3, 1.2	3.92 dd, J = 8.0, 5.5
14	4.03 dt, J = 9.7, 3.2	4.19 dd, J = 5.8, 4.6	4.12 ddd, J = 6.2, 4.4, 1.2	4.19 dd, J = 5.8, 4.4
17	1.88 dd, J = 13.3, 9.9	2.62 dd, J = 14.0, 5.8	2.48 dd, J = 13.6, 6.2	2.73 dd, J = 14.0, 5.6
	3.09 dd, J = 13.5, 3.8	2.97 m	2.84 dd, J = 13.6, 4.3	3.03 dd, J = 14.1, 4.6
19	6.84 m	7.09 m	7.05 m	7.15 m
20	7.17–7.31 m	7.33 m	7.32 m	7.32 m
21		7.33 m	7.32 m	7.32 m
22		7.33 m	7.32 m	7.32 m
23	6.84	7.09 m	7.05 m	7.15 m
CH_3(N-1)				3.86 s
CH_3(N-12)	3.09 s	2.86 s		2.88 s
CH_3(N-15)		2.76 s		2.78 s

[a] Recorded in $CDCl_3$. [b] J values in hertz.

Table IV. ^{13}C NMR Spectral Assignments for Compounds 1, 4, and 7

carbon	1	4	7
2	132.51 (d)	132.01	136.03
3	110.52 (s)	110.31	109.38
4	143.64 (s)	143.64	143.90
5	119.23 (d)	119.13	118.46
6	120.98 (d)	120.98	121.18
7	118.56 (s)	118.36	117.10
8	119.78 (s)	119.80	120.25
9	141.15 (s)	141.10	141.10
10	33.51 (t)	33.04	33.40
11	64.56 (d)	64.53	65.21
13	166.81[a] (s)	166.83[b]	167.05[c]
14	88.03 (s)	87.81	64.87 (d)
16	168.33[a] (s)	168.19[b]	167.57[c]
17	45.43 (t)	43.72	39.12
18	137.37 (s)	136.01	137.92
19	118.40 (d)	131.71	130.90
20	159.11 (s)	130.00 (d)	129.98 (d)
21	115.84 (d)	128.76	128.42
22	131.19 (d)	130.00	129.98
23	122.73 (d)	131.71	130.90
CH_3(N-1)			33.23
CH_3(N-12)	28.45 (q)	28.46	31.77
CH_3(N-15)	34.20 (q)	33.97	33.60

[a–c] Assignments may be interchanged.

this compound also represents the entity from which other members of the group (i.e., compounds 1, 2, 4, and 5) are most likely derived.

It is unfortunate, but natural products bearing nitro groups are relatively rare in nature, and thus only a small number have been studied. In the limited number of investigations wherein the biosynthetic origin of a nitro group was explored, it had been shown to be derived from the oxidation of an amino group rather than by direct nitration. However, in a recent study involving production of the antibiotic dioxapyrrolomycin by *Streptomyces fumanus*, Carter et al. (1989) were able to demonstrate that introduction of the nitro group proceeded in a manner analogous to electrophilic nitration of aromatic compounds by NO_2^+. In the present case, the nitration sequence has not yet been elaborated. However, the unusual presence of a nitro group in the tryptophan indole ring merits classification of the thaxtomins as new variants of microbially produced substances.

After the five phytotoxins had been isolated and identified, investigations were initiated to further our understanding of the structural requirements for the observed phytotoxicity of this class of compounds. As previously demonstrated (King et al., 1989), the presence of a nitro group in the indole ring of the tryptophan moiety and an L,L configuration of the diketopiperazine ring appeared to be the minimal requirements for phytotoxicity. Left to be determined was any prerequisite for the phenyl portion of phenylalanine and how alternate positionings of the nitro group in the indole ring would affect bioactivity. For this purpose the 2,5-dioxopiperazines cyclo-(L-5-nitrotryptophyl-L-phenylalanyl), cyclo-(L-6-nitrotryptophyl-L-phenylalanyl), cyclo-(L-7-nitrotryptophyl-L-phenylalanyl), and cyclo-(L-4-nitrotryptophyl-L-leucyl) were prepared. Subsequent examination of these ana-

logues for phytotoxicity toward aseptically cultured minitubers proved negative in all cases. It is thus apparent that the phenyl portion of phenylalanine plays a necessary part in the structural requirements for phytotoxicity and that the positioning of the nitro group in the indole ring of tryptophan is very site specific.

Once the thaxtomins had been established as phytotoxins associated with the host–parasite relationship of *S. scabies* on potatoes (King et al., 1991), we attempted to determine any similar relationships in other crops susceptible to the scab organism. For this purpose pathogenic isolates of *S. scabies* were grown on the tissue of other appropriate root crops such as sweet potatoes, beets, carrots, and turnips. To date, only in the extracts of infected tissue from turnips has the presence of thaxtomin A been definitively confirmed. The lack of more positive results may have been due partly to the difficulty encountered in infecting the alternate crop tissues. This host range challenge, while somewhat limited, does imply a probable wider extent of the pathogen–phytotoxin relationship for *S. scabies*.

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