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CHICKPEA BLIGHT: PRODUCTION OF THE PHYTOTOXINS SOLANAPYRONES A AND C BY *ASCOCHYTA RABIEI*

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Key Word Index—*Alternaria solani*, *Ascochyta rabiei*, *Cicer arietinum*, Leguminosae, chickpea; blight, phytotoxin, solanapyrone, NMR; mass spectra; X-ray.

Abstract—Filtrates from 12-day-old stationary cultures of *Ascochyta rabiei* grown on Czapek–Dox medium, supplemented with an extract of chickpea seed, killed the cells in cell suspensions obtained by enzymic digestion of chickpea leaflets. Two toxins were isolated by solvent partitioning with ethyl acetate and flash chromatography of the organic fraction on silica Mass spectrometry, UV, $^{13}C/^{1}H$ NMR and X-ray analysis showed that the two compounds were identical to the phytotoxins solanapyrones A and C isolated previously from culture filtrates of the fungus *Alternaria solani*

INTRODUCTION

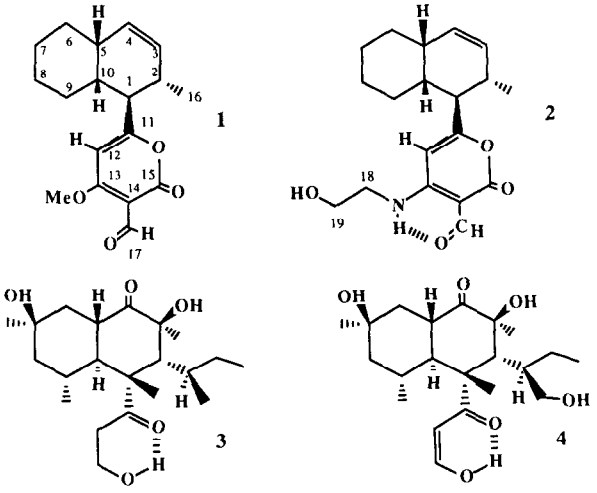
Blight caused by *Ascochyta rabiei* is the most important disease of chickpea in areas where the growing season coincides with cool, moist weather [1]. Serious losses have been recorded around the Mediterranean basin and particularly in Pakistan where, in three successive years during the early part of the decade, they approached 50% of the crop

Early symptoms of infection on leaflets, petioles and young branches include epinasty and loss of turgor, followed by water soaking As these symptoms are consistent with the action of a toxin, the possibility that compounds with toxic activity could be synthesized by the fungus *in vitro* was investigated.

RESULTS

Culture filtrates of *A rabiei* grown on a medium consisting of Czapek–Dox constitutents supplemented with an aqueous extract of chickpea seed were toxic to isolated leaf cells of chickpea When the chickpea extract was omitted no toxic activity was recovered Activity could be partitioned quantitatively into ethyl acetate from filtrates adjusted to pH 3 with hydrochloric acid. Flash chromatography of the ethyl acetate fraction on silica yielded two pure compounds when eluted with hexane–ethyl acetate (1:1) containing 0.1% acetic acid.

Toxin 1 (**1**) was obtained as an oil, UV λ_{max}^{EtOH} nm (ε): 230 (9500), 326 (9700). High resolution mass measurements (70 eV/180° source temp) revealed a diagnostic peak at *m/z* 153 ($C_7H_5O_4$) indicating retention of all the oxygen in a C_7 fragment, a complementary fragment at *m/z* 149 ($C_{11}H_{17}$) retained the charge to a lesser extent (Table 1). These results were more clearly apparent upon examination of the low eV spectra and when taken with the UV data pointed to the presence of an α-pyrone fused to a methyl decalin system.



Toxin 2 (**2**), obtained as crystals, differed from toxin 1 only by substitution of an ethylamino function for the methoxy group. UV λ_{max}^{EtOH} nm (ε): 238 (20 200), 283 (6200), 322 (7800); $[\alpha]_D = -55.6°$ ($CHCl_3$; *c* 0 62) Examination of the $^{13}C/^{1}H$ NMR spectra in $CDCl_3$ showed that toxins 1 and 2 were identical to the known phytotoxins solanapyrones A and C [2].

We chose to assign the spectral data more completely by applying 2D-correlation methods. NOE results served to confirm the assignments reported in Table 2 and indicated that in solanapyrone C the amino hydrogen is hydrogen-bonded to the aldehyde oxygen; the aldehyde proton was clearly very remote from any other protons in both compounds. In solanapyrone A the methoxy group and H-12 interacted strongly through space. The observation of hydrogen bonding was confirmed upon examination of the X-ray structure of solanapyrone C (Fig 1).

Table 1 MS data for toxins 1 and 2

Toxin 1			Toxin 2		
m/z	Formula	Loss	*m/z*	Formula	Loss
302	$C_{18}H_{22}O_4$		331	$C_{19}H_{25}N_1O_4$	
287		Me	316		Me
274	$C_{17}H_{22}O_3$	CO	303		CO
245	$C_{16}H_{21}O_2$	C_2HO_2	300		CH_2OH
207	$C_{11}H_{11}O_4$	C_7H_{11}	286	$C_{18}H_{24}NO_2$	CHO_2
181	$C_9H_9O_4$	C_9H_{13}	273		
153	$C_7H_5O_4$	$C_{11}H_{17}$	259	$C_{16}H_{21}NO_2$	$C_3H_4O_2$
149	$C_{11}H_{17}$	$C_7H_5O_4$	182	$C_8H_8NO_4$	$C_{11}H_{17}$
91	C_7H_7	$C_{11}H_{15}O_4$	152/1		

Table 2 NMR data (500 MHz ^{1}H, 125 8 MHz ^{13}C in $CDCL_3$ solution) of toxins 1 and 2

	δ_c		δ_h†	δ_h†
C*	1	2	1	2‡
16	20 3	20 2	0 95 *d* (6 8)	0 95 *d* (7 0)
8	21 0	21 0	1 18 *m*, 1,47 *m*	1 15–1 27 *m* 2H
				1 45-1 52 *m* 2H
7	25 9	25 9	1 25 *m*, 1 71 *m*	1 71 *m* 1H
9	28 4	28 3	1 41 *m*, 1 48 *m*	1 44 *m* 1H
6	29 7	29 6	1 11 *m*, 1 74 *m*	1 11 *m*, 1 69 *m*
2	35 2	34 6	2 63 *m*, (6 8, 10 0)	2 59 *m*
10	36 0	35 4	2 32 *m*, (11 6)	2 27 *m*
5	36 8	36 7	2 15 *m* (5 0, 1 8)	2 13 *m*
1	48 0	47 3	2 48 *dd* (10.0, 11 6)	2 37 *dd* (10 0, 11 5)
	57 7		4 10 *s* (OMe)	3 2 *br* (OH)
12	95 8	96 0	6 15 s	6 02 s
14	101 8	94 8		
3	130 0	130 3	5 44 *ddd* (10 0, 1 8, 2 0)	5 43 *ddd* (10.0, 1 8, 2 0)
4	131 5	131 4	5 67 *ddd* (10 0, 2 5, 5 0)	5 65 *ddd* (10 0, 2 5, 5 0)
15	162 4	160 5		10 78 *t* (NH)
11	173 6	164 3		
13	176.4	172 2		
17	186 4	191 1	10 15 *s*	9 92 s
18		45 0		3 53 *q* 5 5, 5 0
19		60 7		3 89 *t* 5 5

*Numbering based on ref [2] Shifts w r t $CDCl_3$ at δ77 0

†Shifts w.r t. $CDCl_3$ at δ7 27

‡Not all protons on C-7, C-8 and C-9 are to be taken as rigorously assigned, through lack of 2D-correlation results

The acid lability of solanapyrone C was detected in aged samples dissolved in chloroform-d_1, partial conversion to a diastereoisomeric product was observed which appeared to be epimeric at C-1. Solanapyrone A was about four times as active as solanapyrone C and chickpea cultivars varied in their sensitivity to both compounds (Table 3)

DISCUSSION

Matern *et al.* [3] reported the isolation of two toxins from culture filtrates of *Alternaria solani*, the casual agent of early blight of potato. In common with the present investigation, the fungus was grown on Czapek–Dox liquid medium supplemented with an extract of its host (i.e., potato) In our experiments, we found no toxin in culture filtrates of *A rabiei* when chickpea extract was omitted from the medium, suggesting that it contains a toxin inducing substance. Extracts of host plants have previously been reported to induce toxin production by plant pathogenic fungi *in vitro* [4] and in one instance the inducing compound has been identified [5] Matern *et al* [3] did not characterize the toxins from *A solani* but they gave R_f values for the compounds chromatographed on

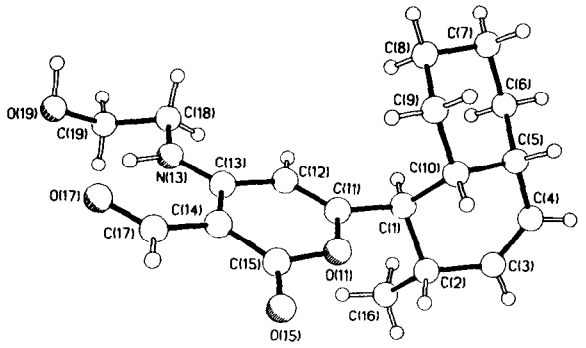


Fig 1 The molecular structure of toxin 2 showing the atom numbering There is an intramolecular hydrogen bond between N(13) O(17), 2 69 Å, H(13) O(17), 1 86 Å, N–H O angle 141° There is an intermolecular hydrogen bond between the hydroxy hydrogen H(19) and the carbonyl oxygen O(15'), 2.76 Å, H(19) O(15'), 1 78 Å, O-H . O angle 171°

Table 3 Sensitivity of cells of various cultivars of chickpea to toxins 1 and 2 of *A rabiei*

Variety tested	Concentration required to give LD_{50} value (μM)*	
	Toxin 1	Toxin 2
CM-1	3.74	14 16
ILC-200	4 80	20 41
NEC-138-2	6 39	35 49
ILC-202	7 59	58 31
ILC-629	8 62	48 02
C-727	9 34	30 23
ICC-5127	9 79	48 02
CM-72	10 29	37 68
ILC-195	10 47	30 23
CM-68	10 66	40 82
C-141	12 02	32 22
C-235	12 39	33 09
RC-32	13 44	54 42
ILC-3279	14 63	39 50
ICC-11514	15 18	42 96
C-44	17 10	74 21
Average	10 40	39 99

*Values are the mean of three replicates

silica gel in six solvents. When solanapyrone A and C were chromatographed in five of these solvents only three of the 10 R_f values fell within 0.1 of those reported [3] These authors also found that their compounds were synergistic when tested on potato; in contrast we found that the effect of solanapyrone A and C on isolated cells was additive rather than synergistic The solanapyrones share interesting structural similarities to several phytotoxins such as the betaenones A and B (**3**) from *Phoma betae*, a parasite of sugar beet [6] and stemphyloxin (**4**) from *Stemphylium botryosum* the causal agent of leaf spot of tomato [7]. The latter compounds can act as lipophylic siderophores which may disturb the iron metabolism of the host. It seems unlikely that the solanapyrones will act directly as iron chelators *per se*, although they may function to chelate iron or other essential metal ions upon ring opening of the pyrone or upon further biotransformation within the host.

The similarity of solanapyrone A to certain acyl tetronic acid ionophores is noteworthy, C-13 is susceptible to nucleophilic attack in the six-membered ring just as the enol of an acyl tetronic acid can undergo conjugate addition/elimination reactions in the five-membered ring.

EXPERIMENTAL

Fungus Isolate 29 of *Ascochyta rabiei* (Pass) Lab was obtained from the National Agricultural Research Centre, Pakistan A loopful of spore suspension in sterile distilled water was streaked onto 2% water agar and incubated at 20° for 48 hr. Colonies from single spores were transferred to chickpea seed agar (2% water agar containing the aq extract of 60 g chickpea seed/l) Inoculum was further multiplied on chickpea seed [8]. After incubation at 20° for 7–10 days the inoculated seed was agitated with 10% sterile glycerol and the resulting suspension adjusted to 10^7 spores/ml. The suspension was distributed to 1.8 ml ampoules in 1 ml aliquots which were stored in liquid N_2

Toxin production and purification Czapek–Dox liquid medium (100 ml/Roux bottle supplemented with an aq. extract of 60 g chickpea seed/l and adjusted to pH 6 4) was inoculated with 1 ml spore suspension (10^7 spore/ml) of *A. rabiei*. The bottles were incubated horizontally, to provide maximum surface area for the culture at 20° in a lighted incubator. After incubation for 12 days the fungus was removed by successive filtration through nylon mesh (80 μm) and glass fibre paper (Whatman GF/A) The filtrate was adjusted to pH 3.0 and partitioned (× 3) against EtOAc The combined EtOAc fractions were dried over Na_2SO_4 and subjected to flash chromatography on a column of silica gel 60 (230–400 mesh, Merck 26 0 × 2 0 cm diam) [9] The column was eluted with hexane–EtOAc (3.1, 600 ml) containing 0 1% HOAc followed by hexane–EtOAc (1 1, 600 ml) containing 0 1% HOAc The eluate from the latter was collected in 30 × 20 ml fractions to separate the two toxins. All fractions were flash evaporated and dissolved in holding buffer before assay [10]

Assay Cells were isolated from the leaflets of 10-day-old chickpea seedlings in an enzyme cocktail and used to assay cultural filtrates and fractions derived from them as previously described [10]

Crystal data $C_{19}H_{25}NO_4$, $M_r = 331\,4$, orthorhombic, $a = 10.616$ (6), $b = 10\,649$ (4), $c = 15\,902$ (8) Å, $U = 1798$ Å^3, space group $P2_12_12_1$, $Z = 4$, $D_c = 1\,22$ g/cm^3, Cu radiation, $\lambda = 1.54178$ Å, $\mu(CuK_\alpha) = 7$ cm^{-1}, $F(000) = 712$. Data was measured on a Nicolet R3m diffractometer with Cu-K_α radiation (graphite monochromator) using ω-scans Independent reflections 1409 were measured ($2\theta \leqslant 116°$), of which 1261 had $|F_o| > 3\sigma(|F_o|)$ and were considered to be observed The structure was solved by direct methods The non-hydrogen atoms were refined anisotropically The protons on N(13) and O(19) were located from a ΔF map and refined anisotropically The positions of the remaining hydrogen atoms were idealised, C–H = 0 96 Å, assigned isotropic thermal parameters, $U(H) = 1\,2\ U_{eq}$ (C), and allowed to ride on their parent carbon atoms Refinement was by block-cascade, full-matrix least squares to $R = 0.046$, $R_w = 0.047$ Computations were carried out on a Eclipse S140 computer using the SHELXTL program system Atomic coordinates, bond lengths and angles have been deposited with the Cambridge Crystallographic Data Centre

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