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SELECTIVE TOXICITY OF WYERONE AND OTHER PHYTOALEXINS TO GRAM-POSITIVE BACTERIA

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Key Word Index—Phytoalexins; antibacterial activity; selective toxicity.

Abstract—The antibacterial activities of isoflavonoid (kievitone and phaseollin), flavonoid (hydroxyflavans), furanoacetylenic (wyerone), and sesquiterpenoid (capsidiol and rishitin), phytoalexins against eight Gram-negative and six Gram-positive bacteria were examined using the paper-disc antibiotic assay method. With the exception of capsidiol, which was inactive at the highest concentration tested (200 μg/disc) all of the phytoalexins were selectively toxic towards Gram-positive species. Wyerone and kievitone were generally more toxic than other phytoalexins; rishitin was the least active inhibitor.

INTRODUCTION

Phytoalexins are generally recognized as fungitoxic compounds synthesized by plants in response to fungal infection [1–3]. Although they may also accumulate in certain plants challenged with bacteria, their antibacterial activity and consequently their role as determinants of bacterial disease reactions remains uncertain [4–10]. Many different classes of compounds have been identified as phytoalexins, but studies on their antibacterial activities have largely been confined to isoflavonoids from legumes, and the sesquiterpenoid rishitin from members of the Solanaceae [5, 9]. Gnanamanickam and Smith [10] recently demonstrated that the isoflavonoid phytoalexins phaseollin, phaseollidin, phaseollinisoflavan and kievitone were selectively toxic to Gram-positive bacteria. This paper reports further studies on the activity of the isoflavonoids, phaseollin and kievitone, using several bacteria not examined in the earlier work [10] and a comparison of their activities with those of flavonoid (hydroxyflavans), furanoacetylenic (wyerone) and sesquiterpenoid (capsidiol and rishitin) phytoalexins [11–13].

RESULTS AND DISCUSSION

The activities of 50 μg of the phytoalexins and of streptomycin (Sigma) were compared against six Gram-positive and eight Gram-negative bacteria including saprophytes and plant and animal pathogens (Table 1). Streptomycin was clearly the most active compound under the assay conditions employed, inhibiting all bacteria except *Proteus rettgeri*. In addition to the isoflavonoids, wyerone and the hydroxyflavans were selectively toxic to Gram-positive bacteria. The sesquiterpenoids failed to inhibit any of the species tested. Kievitone and wyerone were generally the most active of the phytoalexins, an interesting exception being the high activity of 7-hydroxyflavan against *Corynebacterium fascians* (Table 1).

The activities of the sesquiterpenoids were further examined against six bacteria at the higher concentration of 200 μg/disc. Rishitin was found to be selectively toxic, producing small inhibition zones in tests against the Gram-positive *Bacillus megaterium* and *Micrococcus lysodeikticus* (22 and 75 mm^2, respectively), but failing to inhibit the Gram-negative *Erwinia* and *Xanthomonas* species listed in Table 1. Capsidiol was still inactive against these bacteria.

Wyerone was particularly active against the Gram-positive species, concentrations of at most 4 μg producing clear zones of inhibition (Table 2, Fig. 1). By contrast *Pseudomonas syringae* and *Erwinia carotovora* var. *atroseptica* were not inhibited by 300 μg wyerone/disc. The relationship between area of inhibition zone and wyerone concentration was examined with *B. megaterium*. Areas increased with concentration until 30 μg was applied to discs but were not enlarged by further addition of the phytoalexin (Fig. 1).

In view of the sparing solubility of wyerone in water [14], it was considered that the ability of the phytoalexin to diffuse into soft agar and cause inhibition zones may have been facilitated by the presence of traces of 'carrier' methanol remaining in the discs. This possibility was examined by applying 12.5 μg of wyerone to discs in the more volatile solvents acetone and diethyl ether. The solvents used did not markedly affect the size of inhibition zones; mean areas of 105, 76 and 105 mm^2 were recorded from two experiments with acetone, diethyl ether and methanol, respectively.

With the exception of capsidiol, which was inactive at the highest concentrations tested, the paper-disc assay demonstrated the selective toxicity of all the phytoalexins to Gram-positive bacteria. Although many antibiotics are less active against Gram-negative than Gram-positive species, their differential toxicity is often less marked than that of the phytoalexins [15, 16]. Comparable selectivity

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Table 1. Toxicity of 50 μg of phytoalexins and streptomycin sulphate to Gram-negative and Gram-positive bacteria

Bacterium*	Source	Gram reaction	Area of inhibition zone (mm^2)†								
			Kievitone	Phaseollin	Wyerone	Capsidiol	Rishitin	7-Hydroxy-flavan	7,4′-Dihydroxy-flavan	7,4′-Dihydroxy-8-methyl-flavan	Streptomycin sulphate
Erwinia carotovora var. *atroseptica* (PP)	G. D. Lyon‡	−	0	0	0	0	0	0	0	0	212
E. carotovora var. *carotovora* (PP)	NCPPB968§	−	0	0	0	0	0	0	0	0	302
Proteus rettgeri (AP)	SCC‖	−	0	0	0	0	0	0	0	0	0
Pseudomonas phaseolicola (PP)	NCPPB 1321	−	0	0	0	0	0	0	0	0	483
P. syringae (PP)	NCPPB 281	−	0	0	0	0	0	0	0	0	352
Vibrio anguillarum (AP)	SCC	−	0	0	0	0	0	0	0	0	214
Xanthomonas phaseoli (PP)	NCPPB 2064	−	0	0	0	0	0	0	0	0	483
X. phaseoli var. *vignicola* (PP)	NCPPB 2059	−	0	0	0	0	0	0	0	0	302
Bacillus megaterium (S)	SCC	+	137	50	139	0	0	29	22	67	302
Corynebacterium betae (PP)	G. D. Lyon	+	302	126	126	0	0	270	58	173	727
C. fascians (PP)	NCPPB 1675	+	270	89	172	0	0	610	50	120	776
Micrococcus lysodeikticus (S)	SCC	+	149	46	110	0	0	22	0	25	727
Mycobacterium phlei (S)	SCC	+	105	25	90	0	0	32	10	39	633
Streptomyces scabies (PP)	NCPPB 2537	+	105	67	67	0	0	35	43	58	907

* (AP), animal pathogen; (PP), plant pathogen; (S), saprophyte.

† Area of inhibition = area of total inhibition − area of disc; each value is the integer mean of two experiments, no zones of inhibition developed around control discs.

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§ National Collection of Plant Pathogenic Bacteria, Harpenden, Herts.

‖ Stirling University Culture Collection.

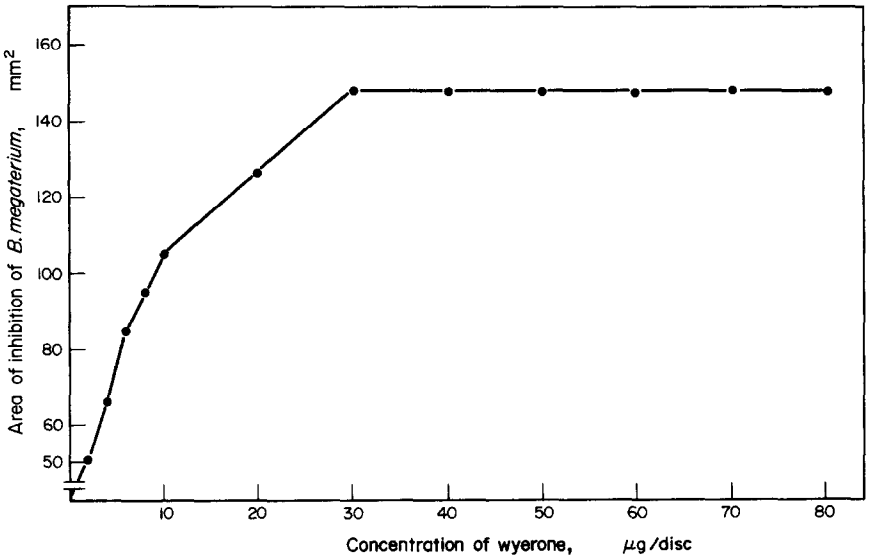

Fig. 1. Relationship between wyerone concentration and area of inhibition of *Bacillus megaterium*.

Table 2. Toxicity of low concentrations of wyerone to Gram-positive bacteria

Bacterium	Area of inhibition (mm^2)*				
	2 μg	4 μg	6 μg	8 μg	10 μg
Corynebacterium betae	50	67	85	105	149
C. fascians	35	67	67	85	105
Micrococcus lysodeikticus	0	22	36	50	57
Mycobacterium phlei	61	105	105	126	149
Streptomyces scabies	0	5	50	50	85

* Calculated as in Table 1.

has been reported, however, for lipophilic tetracyclines, decreased Gram-negative activity being directly associated with increasing lipid solubility [16]. In view of the lipophilic nature of the phytoalexins it is possible that a common cause underlies the inactivity of the compounds against Gram-negative bacteria, perhaps failure to penetrate the outer membrane of their complex cell wall. Whatever the cause of their selectivity, the groups of phytoalexins tested are more likely to have a role in the resistance of plants to Gram-positive than to Gram-negative bacteria.

EXPERIMENTAL

Wyerone was isolated from broad bean cotyledons inoculated with *Botrytis fabae* as described in ref. [17]. Dihydrowyerone was separated from the crystalline product by preparative HPLC using an ODS Hypersil (Shandon) column (200 × 8 mm id) and isocratic elution with MeOH–H_2O (9:11). Volumes (30 μl) of MeOH containing 2–4 mg of the phytoalexins were injected on to the column and wyerone and dihydrowyerone were eluted at 5 ml/min after 45–52 and 58–62 min, respectively [18]. A partially purified sample of rishitin, recovered from potato tuber tissue, was provided by Dr. D. T. Coxon (Food Research Institute, Norwich). After prep. TLC on precoated Si gel plates (Merck 5715) developed in EtOAc–hexane (1:1) rishitin was located at R_f *ca* 0.35 by spraying one edge of chromatograms with vanillin/H_2SO_4 reagent [5, 7]. The untreated phytoalexin was eluted with $CHCl_3$ and its identity confirmed by MS (M^+, 222). Capsidiol and phaseollin were kindly provided by Dr. J. A. Bailey (Long Ashton Research Station, Bristol), and Mr. C. A. Bull and Dr. D. A. Smith (University of Hull) donated the kievitone. Synthetic samples of three phytoalexins from narcissus; 7-hydroxyflavan, 7,4'-dihydroxyflavan and 7,4'-dihydroxy-8-methylflavan were prepared as described in ref. [13]. Concns of kievitone, phaseollin and wyerone were determined spectrophotometrically using published extinction coefficients [11, 19]. Solns of other compounds assayed were prepared from known wts of material.

Antibacterial activity was assessed by the paper-disc method. Antibiotic assay discs (Whatman, 6 mm diameter) were loaded with prescribed amounts of compounds dissolved in 25 μl of MeOH, the solvent alone being added to controls. Discs were dried for at least 1 hr before being transferred to the surface of soft agar plates seeded with the test organisms as previously described [10]. Inhibition zones were measured after incubation for 24 hr at 28°.

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