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# ANTIFUNGAL PHENANTHRENES IN YAM TUBERS

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**Key Word Index**—*Dioscorea rotundata*; Dioscoreaceae; yam; phenols; phenanthrenes; anti-fungal compounds.

**Abstract**—An extract from the peel of yams (*Dioscorea rotundata*) showed anti-fungal activity towards both *Cladosporium cladoporioides* and a variety of yam soft rot pathogens. Two anti-fungal compounds were isolated and identified with the aid of ultra-violet, infra-red, mass and nuclear magnetic resonance spectral data as 2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene (hircinol) and 7-hydroxy-2,4,6-trimethoxyphenanthrene. Neither of the compounds affected the germination of spores of the test fungi, except for hircinol on *Botryodiplodia theobromae* ($ED_{50}$ 63 $\mu$g/ml) whereas both compounds inhibited germ tube growth. The extent of the inhibition varied between species with $ED_{50}$ values ranging from 16 to > 100 $\mu$g/ml. These anti-fungal compounds occur in the skin of the yam where they may function as preformed inhibitors in disease resistance.

## INTRODUCTION

Both preformed and induced anti-fungal compounds can play a role in the resistance of plant roots to fungal infection[1]. Investigations on the susceptibility of yams (*Dioscorea rotundata*) to infection by soft rotting organisms have shown that the soft rot pathogens are wound parasites and do not cause infection through the intact peel of the tuber[2].

Preformed anti-fungal compounds occur in the outer tissues of many vegetable storage organs, e.g. catechol and protocatechuic acid in onion bulbs[3, 4], glycoalkaloids in potato tubers[5] and falcarindiol in carrot roots[6]. Phytoalexins have also been found to accumulate in storage organs challenged by fungi, e.g. hircinol in orchid bulbs[7], rishitin in potato tubers[8] and 6-methoxymellein in carrot roots[9]. The present study was undertaken to investigate whether preformed anti-fungal compounds and phytoalexins are produced by yam tubers and if so to assess their significance in the resistance of yams to soft rotting fungi. We now report the presence of preformed anti-fungal phenolic compounds in extracts of the peel from healthy yam tubers. Two of the three major components of this extract have been identified as known compounds which have been previously isolated from other sources and a third compound tentatively identified as a dihydroxy-dimethoxy-bibenzyl is the subject of further study.

## RESULTS AND DISCUSSION

### *Identifications*

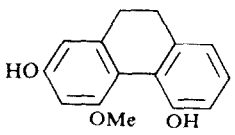
TLC bioassays of the peel extract of yams showed three anti-fungal regions [at $R_f$'s 0.30, 0.43 and 0.68 in solvent system (1)] which corresponded with bands which gave red, brown and purple colours respectively with the diazotized *o*-dianisidine spray reagent. These compounds were either absent or present at much lower concentration in the flesh extract since they were detected neither by spray reagents nor by bioassay against *C. cladosporioides*. Only the two high $R_f$ components have been examined in the study reported here.

High resolution mass spectrometry of the more polar compound **1**, $R_f$ 0.43, which had mp 164–165° (crystallized from $CHCl_3$–hexane), showed a molecular ion of *m/z* 242.0908 indicating the formula $C_{15}H_{14}O_3$. The 90 MHz $^1$H NMR spectrum of **1** ($CDCl_3$–$CD_3OD$) showed signals at $\delta$ 2.64 (4H, *br s*), 3.63 (2H, *br*, $D_2O$-exchangeable, *s*, OH), 3.89 (3H, *s*, OMe), 6.51 (2H, *s*) and 6.77–7.32 (3H, *m*). The 25.2 MHz $^{13}$C NMR spectrum [$(CD_3)_2CO$] showed two methylene carbons at $\delta$31.6 and 31.8, a methoxyl carbon at 57.3, five methine carbons at 100.0, 109.9, 118.2, 120.0 and 128.1 and seven quaternary carbons at 114.7, 128.7, 141.3, 144.1, 154.6, 156.3 and 158.4.

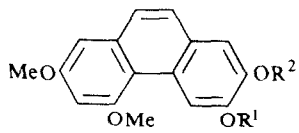
The UV spectrum of **1** exhibited absorptions at $\lambda_{max}$ nm: 213, 272, 292 and 300 and appeared virtually identical to that reported for the compound hircinol[7], a well known orchid phytoalexin. The $^1$H and $^{13}$C NMR and IR spectra were consistent with this structure and the identity was finally confirmed by UV, MS, TLC and HPLC comparison with an authentic sample of synthetic hircinol (kindly provided by Dr. Albert Stoessl). It should perhaps be noted that in their 70 eV EIMS, neither our natural hircinol sample obtained from *Dioscorea rotundata* nor the synthetic sample we obtained gave a mass spectral fragment ion at *m/z* 137 which was regarded as characteristic of hircinol by Fisch *et al.*[10].

The less polar compound **2**, $R_f$ 0.68, which had mp 177–179° (crystallized from methanol) gave a molecular ion at *m/z* 284.1052 corresponding to the formula $C_{17}H_{16}O_4$. **2** gave a very characteristic UV spectrum with absorptions at $\lambda_{max}$ nm: 252 sh, 256 sh, 264, 283 sh, 292 sh, 304 sh, 328, 344 and 361. Both the UV and mass spectra corresponded closely with spectra

reported for 6-hydroxy-2,4,7-trimethoxyphenanthrene (**3**) (batatasin 1), a compound which has previously been isolated from *Dioscorea batatas* [11]. A direct comparison with an authentic sample of synthetic batatasin 1 (**3**) (kindly provided by Dr. Tohru Hashimoto) indicated a very close similarity (UV, MS and TLC comparison) but the two compounds were clearly distinguishable by mp, HPLC and their $^1H$ (Table 1) and $^{13}C$ (Table 2) NMR spectra. The similarity of the NMR spectra (Tables 1 and 2) did indicate however that batatasin 1 (**3**) and **2** have the same 2,4,6,7-oxygenation pattern. This was confirmed by methylating **2** with dimethyl sulphate and potassium carbonate in anhydrous acetone to give 2,4,6,7-tetramethoxyphenanthrene (**4**)[12] identified by MS [(M]$^+$ 298), mp 139–140° (crystallized from methanol) and its $^1H$ NMR spectrum (Table 1). The $^1H$ NMR spectra allow a structure to be assigned to **2** from the three remaining isomeric trimethoxyphenanthrol derivatives. Comparison of the chemical shifts of the aromatic protons H-1, H-3 and H-8 in **2**, **3** and **4** indicates that **2** is 7-hydroxy-2,4,6-trimethoxyphenanthrene which is a known compound with reported mp 177–179°[13]. The $^1H$ NMR spectrum of this compound has also been reported[13] and allowing for a constant shift difference ($\delta$0.10) is in agreement with our spectrum. Based on the isolated yields of **1** and **2** from yam tissue the minimum concentrations of the compounds which must have been present in the peel were 20 $\mu$g/g and 45 $\mu$g/g, respectively.




**1**




**2** $R^1 = Me$, $R^2 = H$

**3** $R^1 = H$, $R^2 = Me$

**4** $R^1 = R^2 = Me$

*Anti-fungal activity*

Neither of the two compounds inhibited germination of the spores of any of the fungi except for **1** against *Botryodiplodia theobromae* ($ED_{50}$ 63 $\mu$g/ml). However both compounds affected germ tube growth and the $ED_{50}$ values are shown in Table 3. Hircinol is reported to exhibit *in vitro* anti-microbial activity against a range of micro-organisms and to behave as a phytoalexin in orchid tubers [7, 14]. However the anti-microbial activity reported by Gäumann[14] is much greater than that reported by Urech *et al.*[7] for the same species despite the fact that both groups used a diffusion test system. For example Gäumann reports hircinol to be lethal to *Aspergillus niger* at 30 $\mu$g/ml while Urech *et al.*[7] found concentrations greater than 500 $\mu$g/ml were necessary to completely inhibit this fungus. Similar discrepancies were also reported for *Trichophyton* species and *Endomyces albicans* as well as for the bacteria *Staphylococcus aureus* and *Escherichia coli*. Our results appear to be intermediary to the above data although we used a different test system. We believe this is the first report of the occurrence of hircinol in *Dioscorea* species where we have found it as a preformed anti-fungal compound. Further studies to find whether it accumulates in response to infection in yams are in progress.

7-Hydroxy-2, 4, 6-trimethoxyphenanthrene (**2**) has been previously isolated from *Combretum psidioides* [13] and *Tamus communis* (Dioscoreaceae) [15] but to our knowledge this is the first report of its isolation from a *Dioscorea* species although the isomeric 6-hydroxy-2,4,7-trimethoxyphenanthrene (batatasin 1) occurs in *Dioscorea batatas* [11] and has growth inhibitory activity. Careful examination of the HPLC chromatograms and the $^1H$ and $^{13}C$ NMR spectra of our crude isolated **2** would suggest that it contains batatasin 1 as an impurity. However the quantity of (**3**) present was very small which may explain why previous workers have failed to detect batatasin 1 in *Dioscorea rotundata* [16]. The anti-fungal activity of these compounds does not appear to have been previously investigated and our results show 7-hydroxy-2, 4, 6-trimethoxyphenanthrene (**2**) to have similar activity to hircinol against some species.

## EXPERIMENTAL

UV spectra were determined in EtOH. IR spectra were determined on KBr discs. NMR spectra were determined in the solvents indicated using TMS as int. standard. Analytical HPLC was carried out on a 25 × 0.46 cm Partisil 5 column eluted with hexane–$CH_2Cl_2$–MeOH–HOAc (50 : 49 : 0.5 : 0.5) at a flow rate of 2 ml/min monitoring by UV absorbance at either 272 nm (compound **1**) or 264 nm (compounds **2** and **3**).

Table 1. $^1H$ NMR spectra of phenanthrene derivatives

| | H-1 or H-3 | H-3 or H-1 | H-5 | H-8 | H-9 or H-10 | H-10 or H-9 | OMe | OH |
|---|---|---|---|---|---|---|---|---|
| Batatasin 1 (**3**) | 6.77 (*d*, *J* = 2 Hz) | 6.89 (*d*, *J* = 2 Hz) | 9.11 *s* | 7.21 *s* | 7.49 (*d*, *J* = 8 Hz) | 7.62 (*d*, *J* = 8 Hz) | 3.93<br>4.02<br>4.08 | 5.85 *br s* |
| **2** | 6.78 (*d*, *J* = 2 Hz) | 6.92 (*d*, *J* = 2 Hz) | 9.10 *s* | 7.35 *s* | 7.51 (*d*, *J* = 8 Hz) | 7.63 (*d*, *J* = 8 Hz) | 3.96<br>4.09 (6H) | 5.94 *br s* |
| **4** | 6.75 (*d*, *J* = 2 Hz) | 6.88 (*d*, *J* = 2 Hz) | 9.08 *s* | 7.20 *s* | 7.51 (*d*, *J* = 8 Hz) | 7.63 (*d*, *J* = 8 Hz) | 3.94<br>4.02<br>4.08 (6H) | — |

Chemical shifts expressed as $\delta$ values for solutions in $CDCl_3$; spectra determined with Perkin Elmer R32 90 MHz instrument.

Table 2. $^{13}C$ NMR spectra of phenanthrene derivatives

| | OMe | CH | C |
|---|---|---|---|
| Batatasin 1 (3) | 55.6<br>56.0(×2) | 99.6, 102.2, 109.1,<br>113.4, 125.2, 128.5 | 115.9, 126.4, 127.6, 135.8,<br>147.1, 147.4, 158.6, 160.3 |
| 2 | 55.5<br>55.9<br>56.1 | 99.7, 102.3, 109.7,<br>112.4, 125.6, 127.9 | 116.4, 125.2, 128.4, 135.4,<br>146.2, 148.5, 158.3, 159.9 |

Chemical shifts expressed as $\delta$ values for solutions in $(CD_3)_2CO$; spectra determined on Bruker WH360 instrument.

Table 3. $ED_{50}$ values ($\mu$g/ml) for phenanthrenes 1 and 2 against germ tube growth

| | *B. theobromae* | *F. moniliforme* | *P. sclerotigenum* | *A. niger* | *B. cinerea* | *C. cladoporioides* |
|---|---|---|---|---|---|---|
| 1 | 16 | 65 | 41 | 42 | 44 | 26 |
| 2 | 79 | 38 | 32 | 14 | > 100 | 59 |

*Preliminary extractions.* Brazilian yams (*Dioscorea rotundata*) were purchased directly from the importer. The tubers were thoroughly washed to remove soil debris and left to dry and only those free from any obvious mechanical damage or infection were selected for use. Peel tissue was obtained by removing *ca* 1 mm thick layer from the outer surface of intact tubers and the remaining inner (parenchymatous) tissue was used as the flesh. Extracts from peel and flesh were prepared separately. 50 g fr. wt each tissue was macerated in peroxide-free $Et_2O$ (500 ml) and the macerate allowed to stand for 30 min before filtering through a sintered funnel. The filtrate was collected and evaporated to dryness under red. pres. at 40° and taken up in 500 $\mu$l cyclohexane–EtOAc (1 : 1) for chromatography. Samples of 10 $\mu$l were applied to 0.25 mm Merck Si gel TLC plates. Plates were developed in equilibrated tanks in the following solvent systems: (1) $CHCl_3$–MeOH (96 : 4); (2) cyclohexane–EtOAc (1 : 1); (3) hexane–$Et_2O$ (2 : 1). Duplicate sets of plates where prepared and developed. One set was first exposed to $I_2$ vapour and subsequently sprayed with diazotized *o*-dianisidine (0.5% in 50% aq. MeOH) followed by ammonia (0.880 $NH_4OH$–MeOH, 1 : 1) to locate spots whilst the duplicates were used for the fungal bioassays.

*Isolation and purification of anti-fungal compounds. Ca* 4 kg yam peel was soaked for 4 days in 7 l. re-distilled peroxide-free $Et_2O$. The $Et_2O$ was decanted and a further 5 l. $Et_2O$ was added to the residue which was soaked for a further 4 days. The combined extracts were filtered and evaporated to a brown tar (3.94 g) which was re-dissolved in 12 ml $CHCl_3$–MeOH (96:4). Triplicate 4 ml aliquots of this soln were separated by flash CC [17] on 40–63 $\mu$m Si columns (Merck 9385) eluting with $CHCl_3$–MeOH (96 : 4) at 35 kPa pressure. Each column was dry packed and measured 14 × 5 cm i.d. Fractions of 20 ml were collected and monitored by TLC using both chemical visualization and the bioassay. Appropriate fractions from the three columns were combined and subjected to prep. TLC on activated (1 hr at 120°) 0.5 mm Si gel plates at a loading of ≤ 50 mg/plate developing in $CHCl_3$–MeOH (96 : 4) or using multiple development in $CHCl_3$. In this way three anti-fungal components were obtained which were chromatographically homogeneous in solvent systems (1)–(3).

*Fungi.* Single strains of the yam pathogens, *Botryodiplodia theobromae, Fusarium moniliforme, Penicillium sclerotigenum* and *Aspergillus niger* together with *Botrytis cinerea* and *Cladosporium cladosporioides* were used. All fungi were grown on Difco Bacto potato dextrose agar slopes or plates at 25° and exposed to near UV light when necessary to stimulate sporulation.

*Preparation of spore suspensions.* Spores of all the fungi were harvested by flooding slopes or plates of the fungus with sterile distilled $H_2O$ containing 0.1% Tween 80 and rubbing the culture with a sterile glass rod. The spore suspensions were then filtered through two layers of sterile muslin to remove mycelial fragments. The filtered spores were centrifuged and washed twice with 10 ml of sterile glass distilled $H_2O$ before adjusting the concn to *ca* $10^5$ or $10^6$/ml according to their future use.

*TLC bioassay techniques.* A similar method to that of Homans and Fuchs [18] was employed. Developed Si gel plates were sprayed with either a spore suspension ($10^6$/ml) of *C. cladosporioides, Aspergillus niger, Penicillium sclerotigenum* or *Botryodiplodia theobromae* in potato dextrose broth. The plates were then incubated in sealed humid chambers at 25° for 4 days in the dark, to locate anti-fungal compounds.

*Spore germination and germ tube growth tests.* A spore suspension of each of the fungi in potato dextrose broth was added to alcoholic solns of the two yam compounds to give a final spore concn of *ca* $10^5$ per ml and 10, 20, 50 and 100 $\mu$g/ml of the two compounds. The final alcohol concn was 10%. After careful mixing 0.2 ml of each treatment was pipetted onto sterile glass microscope slides; the suspension was retained within a rectangle marked on the slides with a grease pencil. The prepared slides were incubated on glycerol agar plates at 25° for 17 hr in the case of *F. moniliforme, P. sclerotigenum, A. niger* and *C. cladosporioides*, 6 hr for *B. cinerea* and 4 hr for *B. theobromae.* After the required period of incubation direct counts were made on the slides to determine the percentage germination (300

spores/slide) and mean germ tube length (50 germ tubes/slide) for each treatment. All treatments were duplicated and each expt carried out twice. The percentage inhibition of germination and germ tube growth was calculated by comparing germination and germ tube growth of spores incubated under identical conditions in the absence of the test compounds. The $ED_{50}$ values were obtained by plotting percentage inhibition against log of the concn and interpolating.

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