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DIHYDROSTILBENE PHYTOALEXINS FROM *DIOSCOREA ROTUNDATA*

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Key Word Index—*Dioscorea rotundata*; white yam; batatasin IV; dihydropinosylvin; *Botryodiplodia theobromae*; phytoalexin.

Abstract—2′,3-Dihydroxy-5-methoxybibenzyl (Batatasin IV), its demethyl derivative and 3,5-dihydroxybibenzyl (dihydropinosylvin) were isolated only from flesh of *Dioscorea rotundata* infected with *Botryodiplodia theobromae* and may therefore be considered phytoalexins. These compounds were found to be antifungal using bioassays with *Cladosporium cladosporioides*, *Botryodiplodia theobromae*, *Aspergillus niger* and *Penicillium schlerotgenum*. Dihydropinosylvinin also exhibited strong antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*.

INTRODUCTION

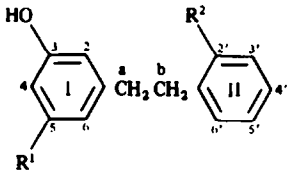
White yam, *Discorea rotundata* is the most important economic tuber in tropical West Africa [1]. As a result of previous work dormancy inducing compounds batatasin I, II, III, IV and V have been isolated from *D. batatas* [2–4] and detected in *Discorea* species other than the white yam [5]. Batatasin I together with hircinol and 7,-hydroxy-2,4,6,-trimethoxyphenanthrene were later found in the peel of *D. rotundata* and implicated as preformed microbial inhibitors in disease resistance [6].

In addition to preformed inhibitors, phytoalexin accumulation in response to infection has been established as a mechanism of plant resistance to disease. Their accumulation under stress or infective conditions in storage organs such as 6-methoxy mellein in carrot [7] and rishitin in potato tuber [8] is well known. This work reports for the first time, the occurrence of phytoalexins in the tuber of *D. rotundata*, although bibenzyl phytoalexins have been recently isolated from *D. batatas* [9] and three compounds, one of which have not been isolated from plants previously are reported.

RESULTS AND DISCUSSION

Diethyl ether extraction of *D. rotundata* infected with *Bothyodiplodia theobromae* showed fungitoxic zones in TLC bioassay against *Cladosporium cladosporioides* using $CHCl_3$–MeOH (24:1) as solvent. Three fungitoxic spots not found in the control samples gave reddish/pink colours with vanillin–H_2SO_4 spray [3]. The extract was fractionated by column chromatography over silica gel (mesh 60–120) and purified by TLC to give compounds **1**–**3**.

Compound, **1**, had a UV λ_{max} 280 sh 282 indicating a bibenzyl system. A characteristic mass spectrum with ions



1 R^1 = OH, R^2 = H
2 R^1 = OH, R^2 = OH
3 R^1 = OMe, R^2 = OH

at *m/z* 214 ($[M]^+$ $C_{14}H_{14}O_2$), *m/z* 123 (62%), a hydroxytropylium ion, and *m/z* 91 (100%), a dihydroxytropylium ion, is typical of a bibenzyl system [3, 5]. The ^{1}H NMR spectrum ($CDCl_3$) which contained the characteristic Ar–CH_2–CH_2–Ar four proton multiplet at δ2.79, a broad proton singlet attributable to three nearly equivalent protons in the dihydroxybenzyl ring at δ6.25 and a five proton multiplet centred at δ7.21 due to the protons in an unsubstituted benzyl ring further confirmed this structure. The ^{13}C NMR signals (Table 1) with DEPT are also consistent with the structure of 3,5-dihydroxybibenzyl and this compound was identical with dihydropinosylvin recently isolated from *D. batatas* [9].

Compound **2** possesses a UV λ_{max} 272 sh 275, suggesting another bibenzyl system similar to **1**. The mass spectrum with ions at *m/z* 230 ($[M]^+$, $C_{14}H_{14}O_2$) and *m/z* 123 (25%) and 107 (100%) suggested a compound with an additional hydroxyl group on the unsubstituted benzyl-ring of **1** since the tropylium ion at *m/z* 107 in **2** is 16 mass units greater than that of **1**. The ^{1}H NMR had the characteristic four proton Ar–CH_2–CH_2–Ar signal at δ2.86. There was also a one proton singlet at δ6.26, and a singlet equivalent to two protons at δ6.30. The coupling patterns of the protons at δ6.81 and δ7.06 indicate that the additional hydroxyl substituent is on the benzyl ring that was unsubstituted in **1**. This was consistent with the

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Table 1. Comparison of ^{13}C NMR* spectra of dihydropinosylvin 1 demethyl batatasin IV 2 and batatasin IV 3

Carbon No	Compound 1	2	3
a	37.4	36.1	36.28
b	37.3	31.9	32.01
1	144.9	145.1	144.74
2	108.1	107.72	106.70
3	156.8	157.3	156.77
4	100.5	100.6	99.18
5	156.8	157.3	160.95
6	108.1	107.72	107.98
1'	141.6	128.5	127.81
2'	128.5	154.0	153.61
3'	128.4	115.0	115.42
4'	126.0	127.2	127.37
5'	128.4	120.5	120.85
6'	128.5	130.26	130.32
OMe	—	—	55.23

*Chemical shift in ppm from TMS in $CDCl_3$.

Table 2. Antifungal activity of dihydropinosylvin 1 and demethyl batatasin 2

	Spore germination ED_{80}(μg/ml) 1	2	Inhibition of germ tube elongation ED_{50}(μg/ml) 1	2
A. niger	47	55	46	46
B. theobromae	66	62	81	59
P. sclerotigenum	50	56	46	54
C. cladosporioides	50	49	53	53

Spore concentration was 10^5/ml. Concentrations of the compounds used were 10, 20, 50 and 100 g/ml. Slides were incubated at 25° for 17 hr for *C. cladosporioides*, and *A. niger*; 4 hr for *B. theobromae*. Direct counts were made to determine percentage germination (300/slide) and mean germ tube length (50/slide). ED_{50} values were obtained from a plot of percentage inhibition against log of the concentration and interpolating.

Table 3. Antibacterial activity of dihydropinosylvin 1 and demethyl batatasin 2

	Antibacterial activity 1	MIC (μg/ml) 2
Bacillus cereus	10	100
S. aureus	10	50
P. aurigenosa	10	10
E. coli	10	50

MIC = minimum inhibitory concentration was determined using standard techniques with compound concentrations of 10, 20, 50 and 100 g/ml. The diameter of the inhibitory zone was measured after 24 hr at 25°.

signals observed in the ^{13}C NMR spectrum (Table 1) and was confirmed by DEPT. The compound is thus 2',3,5-trihydroxybibenzyl 2, and has been named demethyl batatasin IV. This compound has not been isolated from plants previously.

The third compound, 3, has a UV spectrum and ^{1}H NMR similar to 2, except for the –OMe signal at δ3.74. The characteristic ions in the mass spectrum at *m/z* 138 (25%) showed that the OMe group is an additional substituent in a hydroxylbenzyl ring. ^{1}H NMR suggested that one benzyl ring of the bibenzyl had OH and OMe substitution and the other benzyl ring contained one OH substituent. Irradiation of the methoxyl signal in an NOE experiment located the OMe as *meta* to the OH and these substituents were therefore assigned as in structure 3. The ^{13}C NMR shifts (Table 1) are consistent with the structure being that of batatasin IV previously isolated as one of the dormancy inducing compounds in *D. batatas* [3].

Antibiotic activities

Available material permitted only the *in vitro* antimicrobial assay of demethyl batatasin IV, 2, and dihydropinosylvin, 1; however, all compounds gave positive results in the TLC bioassay using *C. cladosporioides*. Demethylbatatasin IV and dihydropinosylvin both inhibited spore germination and germ tube elongation of the pathogens *B. theobromae*, *Aspergillus niger*, *Penicillium sclerotigenum* and *C. cladosporioides* at levels (ED_{50} *ca* 50 g/ml) which were comparable to those reported for the preformed antifungal compounds in the peel of the yam tuber [6] (Tables 2 and 3). Dihydropinosylvin, 1, showed a marked antibacterial activity against all the organisms (both Gram positive and Gram negative) tested (Table 3) with an MIC ≤ 10 μg/ml. This activity is common to similar compounds where one of the benzyl rings is unsubstituted [10]. The antibacterial activity of demethyl batatasin IV, 2, was less marked and varied with the organisms tested. From these results and others [10] it would appear that 2'-hydroxylation decreases antibacterial activity. It has been proposed that the biosynthesis of phenanthrenes and dihydrophenanthrenes proceeds via 4' and 2'-hydroxybibenzyl because of their co-occurrence in some families such as *Combretaceae* [11] and *Dioscoraceae* [2, 3]. The bibenzyls may arise from appropriate stilbene precursors by reduction of the double bond of Ar–CH = CH–Ar. The compounds isolated may therefore originate from a stilbene precursor to give dihydropinosylvin, 1, which is hydroxylated to demethylbatatasin IV, 2, before methylation to batatasin IV, 3, which has been proposed as a precursor of hircinol [12].

EXPERIMENTAL

Plant material and phytoalexin induction. D. rotundata Poir. tubers purchased at Ile-Ife, were washed thoroughly with dist. H_2O and peeled. The white flesh was further rinsed with dist. H_2O and cut into 8–10 mm thick pieces. Phytoalexin synthesis was induced by dipping the pieces in a sterile dist. H_2O suspension of *B. theobromae* mycelia, for 10 min before incubation in sterile Petri dishes for a further 30 hr. Control tuber pieces were dipped in sterile dist. H_2O only and similarly incubated.

Extraction and purification of phytoalexins. Induced and control tuber pieces were macerated in cold peroxide-free Et_2O,

and allowed to stand for 30 min before filtration. The marc was re-extracted twice, the Et_2O fractions pooled and the solvent removed *in vacuo* at 30°. Aliquots of the induced and control extracts were chromatographed in duplicate on silica gel TLC plates with $CHCl_3$–MeOH (24:1). Developed chromatograms were air-dried and a set sprayed with a spore suspension of *C. cladosporioides* in potato dextrose broth [5] and incubated for 4 days under moist condition at 25° in the dark to detect antifungal zones. The other set was sprayed with vanillin–H_2SO_4 spray reagent to locate pinkish-red and blue-grey coloured spots not observed in the control at R_f values corresponding to the inhibitory zones on the spore-sprayed plates. The isolation of the compounds giving the blue-grey response is in progress.

The extract from infected yam tuber (3 g) was then subjected to CC using silica gel (mesh 60–120) and eluted successively with 500 ml each of the following solvent mixtures: $CHCl_3$, $CHCl_3$–MeOH, (9:1), (8:2), (7:3) and (1:1). Fractions (25 ml) were collected, monitored by silica gel TLC using $CHCl_3$, $CHCl_3$–MeOH (24:1) to locate compounds **1–3** in fractions 55–63, 64–71 and 72–74, respectively. Fractions containing **1** (R_f 0.30) and **2** (R_f 0.45) were purified by repeated prep. TLC in $CHCl_3$ followed by $CHCl_3$–MeOH (24:1). The fraction containing **3** (R_f 0.18) was purified by CC over silica gel (mesh 60–120) using exane–$CHCl_3$ (1:1).

Spectroscopic data. MS were recorded at 70 eV. ^1H and ^{13}C NMR spectra were taken at 300 MHz in $CDCl_3$ with TMS as int. stnd.

Dihydropinosylvin **1**. Yield 74.1 g/g fr. tuber. UV λ_{MeOH} nm: 280 sh 282; $AlCl_3$: no shift. MS *m/z* (rel. int.): 214 (48) $[M]^+$; 123 (62); 91 (100), 77 (4), 65 (11), 55 (4); ^1H NMR: 2.79 (4H, *dd* J = 12.5, 4.0 Hz Ar–CH_2–CH_2–Ar) 6.25 (3H, *br s*; H-2, H-4, H-6), 7.21 (5H, *m*, H-2′, H-3′, H-4′, H-5′, H-6′).

Demethyl batatasin IV **2**. Yield 56.0 g/g fr. tuber. UV λ_{MeOH}: 267, 272 sh, 275, 282 sh., $AlCl_3$ no shift. MS *m/z* (rel. int.): 230 (19) $[M]^+$, 124 (10) 123 (16) 122 (12) 10 (30) 107 (100) 91 (3) 77 (8); ^1H NMR: δ2.82 (4H, *dd*, J = 13.0, 3.1 Hz Ar–CH_2–CH_2–Ar) 6.26 (1H, *s*, H-4) 6.30 (2H, *d*, J = 2.1 Hz, H-2, H-6) 6.80, (1H, *dd*, J = 8.5, 1.5 Hz, 1H, H-3′) 6.86 (1H, *dd*, J = 8.0, 1.5 Hz, H-6′) 7.10 (2H, *m*, H 4′, 5′).

Batatasin IV **3**. Yield 58.0 mg/g fr. tuber. UV, MS, ^1H NMR data as reported in ref. [3]. NOE, irradiation δ2.96 (–CH_2–) enhancement δ2.81 (–CH_2–) 6.30 (H-2) and 6.36 (H-6) irradiation at δ3.74 (OMe) enhancement at 6.27 (H-4) 6.34 (H-6) irradiation centred at δ7.10, enhancement 6.75–6.86.

Microbes for antimicrobial assay. Single strains of yam fungal pathogen *B. theobromae* (Pat) *Aspergillus niger* var. Teigh, *Penicillium sclerotigenum* 1 (Yamamoto) and *Cladosporium cladosporioides* were used. They were maintained on potato dextrose agar plates at 25°. The bacteria used were *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* maintained on nutrient broth slants at 4°. *Fungal spore germination and germ tube elongation tests.* The method of ref. [5] was used.

Antibacterial tests. Cultures from slants were grown in nutrient broth for 18 hr before spreading 0.2 ml of the mixed suspension evenly on nutrient broth agar plates. Sterile paper discs 0.6 mm diam were loaded with different concns of the test compound (0, 10, 20, 50 and 100 μg/ml) and allowed to air dry before placing on the bacteria seeded agar plates. The diam of the inhibitory zones were measured after 24 hr incubation at 25°. The minimum inhibitory concentration (MIC) was taken as the lowest concn showing zones of inhibition.

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