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# TOXIC CYTOCHALASINS OF *PHOMOPSIS PASPALLI*, A PATHOGEN OF KODO MILLET*

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**Key Word Index**—*Paspalum scrobiculatum*; Gramineae; kodo millet; *Phomopsis paspalli*; fungi; cytochalasins; kodo-cytochalasin-**1**; kodo-cytochalasin-**2**.

**Abstract**—*Phomopsis paspalli*, a fungal pathogen of kodo millet, produces two major metabolites, which have been found to be new members of the recently discovered class of mould products, cytochalasins.

## INTRODUCTION

KODO millet (*Paspalum scrobiculatum* Linn. Syn. *P. commersonii* Lam; Sanskrit, *kodrava*; Hindi, *kodo*; Marathi, *harik*) is a minor grain crop, which, since ancient times, has been cultivated in India, particularly on the coastal areas.[1] The grains have often been reported to cause poisoning of man and animals when used as food and the ancient text, *Arthasastra* (300 B.C.)[2] describes its use as a poison for tigers. The chief symptoms of kodrava poisoning are unconsciousness, delirium with violent tremors of the voluntary muscles, vomiting and difficulty in swallowing.[1] The poisonous part of the grain is said to be the outer coat and a fungus is believed to be involved.[1] The toxic principle, along with lipids, can be extracted by petrol and a method has been developed to distinguish poisonous from the non-poisonous grains; the petrol extract from the poisonous grains gives red colour with conc. $H_2SO_4$, whereas the extract from innocuous grains does not give this colour.[3]

In view of the claim that a fungus is associated with the toxic grains, Pendse *et al.*[4] examined a large number of samples of toxic grains, from the Maharashtra region of India, and found that the samples were always infected with fungi. The predominant fungus belonged to the genus *Phomopsis* and was designated *Phomopsis paspalli*, Pendse and Kanitkar.[4] This paper describes the isolation and characterization of the toxic metabolites of this fungus.

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[1] (1966) *The Wealth of India* (*Raw Materials*), Vol. VII, pp. 270–273, Council of Scientific and Industrial Research, Delhi.

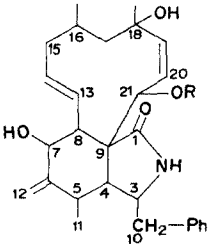
[2] Kautilya *Arthasastra*, *adhi* 4, *adhya* 3, p. 209. English translation by R. Shamasastry (1960), p. 236, Mysore Printing and Publishing House, Mysore.

[3] AYYAR, K. V. S. and NARAYANASWAMY, K. (1949) *Nature, Lond.* **163**, 912.

[4] (a) PENDSE, G. S. (1974) *Experientia*, in press; (b) PENDSE, G. S., KANITKAR, U. K. and DESHMUKH, P. G. (1974) *Ant. Leewenhoek J. Microbiol. Serology*, submitted.

## RESULTS AND DISCUSSION

The fungus was grown as a surface culture on potato–dextrose–agar medium. The alcohol extract of the mycelium together with the medium was successively extracted with petrol., ether, ethyl acetate and acetone. The ether extract showed symptoms typical of kodrava toxicity in dogs. On TLC this extract showed the presence of two major and two minor constituents; the two major constituents were obtained pure by preparation-layer chromatography (PLC). In view of their most plausible structures, these metabolites have been named kodo-cytochalasin-**1** and kodo-cytochalasin-**2** and are new members of the recently discovered class of mould products, cytochalasins.[5] Both compounds give a deep red colour with conc. $H_2SO_4$ (cf. test for poisonous grains, cited above) and, are toxic [e.g. lethal dose of kodo-cytochlasin-**1** in mice: 2 mg/kg (death after 45 min), 5 mg/kg (death after 20 min)].




(**1**) R = Ac
(**2**) R = H

*Kodo-cytochalasin*-**1** (**1**)

The compound analysed for $C_{30}H_{39}O_5N$ ($M^+$, *m/e* 493) and contained one OAc group, IR (1745, 1235 $cm^{-1}$), PMR (3H, *s*, 2·26 ppm), and two OH groups, IR (3300 *broad*, 1018 $cm^{-1}$), PMR (CHOH: 1H, *bd*, 3·83 ppm, $J$ 10 Hz). On acetylation the compound yielded a diacetate (PMR: 3H singlets at 2·27, 1·95 ppm) showing OH absorption IR (3300 $cm^{-1}$); as expected the CHOH doublet of the original compound moved down-field ($\sim$ 5·1 ppm) in the diacetate. Thus, there must be at least two free hydroxyls, of which one is secondary and the other tertiary. IR absorption (1690 $cm^{-1}$) indicated –CONH– with the non-basic. The carbonyl function could not be reduced by $NaBH_4$ suggesting a $\gamma$-lactam.[6]

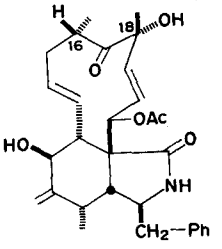
The spectral data also indicated the presence of $>C{=}CH_2$ (IR 918 $cm^{-1}$, PMR, two 1H broad singlets at 5·11 and 5·36 ppm) and HC=CH (IR 970 $cm^{-1}$).

From the PMR spectrum, it is also clear that the metabolite has two $CH_3$–CH< (two overlapping 3H doublets centred at 0·97 and 1·01 ppm, each with $J \simeq 7$ Hz), one $CH_3$–C–O (3H, *s*, 1·33 ppm) and, that it displays a total of seven protons in the region 5·0–6·1 ppm (olefinic protons/C=C–CHOAc[7]) and five protons in the region 7–7·6 ppm (aromatic protons). Irradiation at $\delta$ 2·8 (allylic methines) causes the CHOH doublet (3·83 ppm) and one of the $CH_3$–CH doublets (0·97 ppm) to collapse to broad singlets. This is consistent with the presence of the groupings –C–CHOH–CH(–C<)–C=C– and –C=C–CH–Me respectively.

[5] See e.g. (a) TURNER, W. B. (1971) *Fungal Metabolites*, pp. 352–354, Academic Press, London; (b) BINDER, M., TAMM, C., TURNER, W. B. and MINATO H. (1973) *J. Chem. Soc.* 1146.
[6] BELLAMY, L. J. (1958) *The Infrared Spectra of Complex Molecules*, p. 213, Methuen, London.
[7] See e.g. GUPTA, A. S. and SUKH DEV (1971) *Tetrahedron* **27**, 640.

On catalytic hydrogenation the compound furnished a dodecahydro derivative, $C_{30}H_{51}O_5N$ ($M^+$, *m/e* 505), which gave no colour with tetranitromethane. This information coupled with the other data shows that the metabolite must be tetracyclic with one ring benzenoid. This further requires that there must be present a total of three olefinic bonds and taking into account the number of protons contributing to the signals in the 5–6·1 ppm region, one can infer that the third olefinic linkage also must be disubstituted and that the acetate function must be a secondary alcohol derivative.

With the above information, a search of the literature[5,8,9] showed that the compound is, in all probability, a member of the new class of mould metabolites, cytochalasins, first reported in 1966–1967.[10] A comparison of its PMR spectrum with the spectral data reported[11] for cytochalasins showed several similar features; similarity with the spectrum* of cytochalasin-D[11b] (**3**) (zygosporin-A[12]) is especially striking. In view of this, structure **1**, which meets all the structural requirements of the new metabolite, appears highly probable. This structure is consistent with the shift of the doublet at 1·2 ppm in the spectrum of cytochalasin-D (assignable to $C_{16}$–Me, which is α to C=O) to 1·01 ppm in the PMR spectrum of kodo-cytochalasin-**1**; likewise, $C_{18}$–Me is at 1·50 ppm in cytochalasin-D, whereas it occurs slightly upfield (1·33 ppm), as expected, in the spectrum of the new metabolite. The position and shape of the signals due to C=C$\underline{H}_2$, C$\underline{H}$OH, OCOC$\underline{H}_3$ and the aromatic protons are almost identical in the two spectra.




(3)

Structure **1** is fully supported by its MS which is very similar to that of cytochalasin-D and other cytochalasins.[11] The base peak is at *m/e* 91, and shows intense ions at *m/e* 120 and M-91. The MS of cytochalasin-D and kodo-cytochalasin-**1** are virtually identical in the region *m/e* 50 to *m/e* 250, but above *m/e* 250 several important ions occur 14 a.m.u. lower in the spectrum of kodo-cytochalasin-**1** replacement of C=O by $CH_2$ (*m/e* 493, 433, 415, 402, 342, 324, 314, 296).

It has not been possible to provide a complete chemical proof of the structure of kodo-cytochalasin-**1** because of the lack of a sufficient quantity of the compound.

* Authors are grateful to Dr. H. Minato, Shionogi Research Laboratory, Osaka, Japan, for providing a sample of cytochalasin-D.

[8] MILLER, M. W. (1961) *The Pfizer Handbook of Microbial Metabolites*, McGraw Hill, New York.
[9] SHIBATA, S., NATORI, S. and UDAGAWA, S. (1964) *List of Fungal Products*, University of Tokyo Press, Tokyo.
[10] (a) ALDRIDGE, D. C., ARMSTRONG, J. J., SPEAKE, R. N. and TURNER, W. B. (1967) *Chem. Commun.* 26; (b) ROTHWEILER, W. and TAMM, CH. (1966) *Experientia* **22**, 750.
[11] (a) ALDRIDGE, D. C., ARMSTRONG, J. J., SPEAKE, R. N. and TURNER, W. B. (1967) *J. Chem. Soc.* (*C*) 1667; (b) ALDRIDGE, D. C. and TURNER, W. B. (1969) *J. Chem. Soc.* (*C*) 923.
[12] TSUKUDA, Y., MATSUMOTO, M., MINATO, H. and KOYAMA, H. (1969) *Chem. Commun.* 41.

*Kodo-cytochalasin*-**2** (**2**)

From its spectral data (see Experimental) it is clear that the compound is desacetyl kodo-cytochalasin-**1**. This was confirmed, when hydrolysis of kodo-cytochalasin-**1** furnished a product identical (m.m.p., IR, MS) with kodo-cytochalasin-**2**.

## EXPERIMENTAL

All m.ps are uncorrected. IR spectra were recorded as Nujol mulls; PMR spectrum were taken in $CDCl_3$ at 60 MHz, signals are recorded in $\delta$ (ppm) relative to TMS. MS was determined at 70 eV using a direct inlet system. TLC and PLC were carried out on silica gel layers containing 15% gypsum.

*Isolation of metabolites. Phomopsis paspalli* was cultivated in large conical flasks on 2% potato–dextrose–agar medium. After a growth period of 3 weeks, the entire material, including the medium was agitated, an amount of 95% EtOH sufficient to cover the entire material added and left at 20° for 4 days. The aqueous alcoholic extract was drained off and evaporated under red. pres. The residue (65 g) was mixed with $Na_2SO_4$ (300 g) and extracted, by cold percolation, with the following sequence of solvents to furnish various extracts: light petrol (124 mg, gum), $Et_2O$ (585 mg, yellow sticky mass), EtOAc (1·7 g, yellow solid) and $Me_2CO$ (618 mg, gum).

The $Et_2O$ extract, which was biologically active, showed on TLC (solvent: 10% MeOH in $CHCl_3$, spray conc. $H_2SO_4$) four spots with $R_f$ 0·56 (yellow, minor), 0·49 (red, major), 0·36 (yellow, minor) and 0·32 (red, major). The two major metabolites were separated by PLC. In a typical experiment, 337 mg of the $Et_2O$ extract furnished 132 mg of material $R_f$ 0·49 and 28 mg material $R_f$ 0·32.

*Kodo-cytochalasin*-**1** (*21-acetoxy-7,18-dihydroxy-16,18-dimethyl-10-phenyl*[11]*cyctochalasa-6*(12),13,19-*triene-1-one*). Two crystallizations of the less polar material, from $CHCl_3$–$Et_2O$ yielded white needles, m.p. 258–263°, $[\alpha]_D$ −9·0° ($CHCl_3$, *c* 0·5%), +91·2 (MeOH, *c* 0·1%).* (Found: C, 73·2; H, 8·2; N, 2·83; $C_{30}H_{39}O_5N$ requires: C, 72·99; H, 7·96; N, 2·84%).

*Kodo-cytochalasin*-**2** (*7,18,21-trihydroxy-16,18-dimethyl-10-phenyl*[11]*cyctochalasa-6*(12),13,19-*triene-1-one*). The more polar compound was crystallized twice from $CH_2Cl_2$–petrol. to furnish white needles, m.p. 135–138°, IR (KBr): OH 3200–3500 $cm^{-1}$; C=O 1690 $cm^{-1}$; CH=CH 970 $cm^{-1}$. PMR: two C<u>H</u>$_3$–CH< (3H doublets at 1·03 and 1·09 ppm, each with *J* 7 Hz), C<u>H</u>$_3$–C–O (3H, *s*, 1·33 ppm), two C<u>H</u>OH (1H, *bd*, 3·80 ppm, *J* 12 Hz; 1H, *bs*, 4·06 ppm), >C=C<u>H</u>$_2$ (two 1H broad singlets at 5·10 and 5·31 ppm), four olefinic protons (overlapping multiplet, 5·54–6·10 ppm), and aromatic protons (*m*, 6·90–7·2 ppm). MS *m/e* (rel. intensity) 433 ($M-H_2O^+$, 45), 415 (12), 397 (13), 342 (60), 324 (22), 306 (12), 251 (20), 120 (30), 91 (100).

*Kodo-cytochalasin*-**1**. *Hydrolysis.* To 5% methanolic KOH (1·5 ml), 10 mg of kodo-cytochalasin-**1** was added and left at 20° for 4 hr. The solvent was removed under red. pres., the residue diluted with $H_2O$ and acidified with HCl. The product was extracted with $CHCl_3$, washed with $H_2O$ dried and evaporated to dryness. The residue (10 mg) was crystallized from $CH_2Cl_2$–petrol. to furnish white needles, m.p. 126–130°, m.m.p. with kodo-cytochalasin-**2** was 130–135°.

*Acetylation.* $C_5H_5N$ (1 ml), $(ACO)_2O$ (1 ml) acetylation of kodo-cytochlasin-**1** (30 mg) for 14 hr at 20°, gave after the usual work-up, a solid (m.p. 110–118°) which was crystallized from $CHCl_3$–petrol. to yield white crystals, m.p. 126–130°. IR (KBr): OH 3300 $cm^{-1}$; C=O 1685, 1735 $cm^{-1}$. PMR: two C<u>H</u>$_3$–CH< (3H doublets centred at 0·96 and 1·00 ppm, each with *J* 7 Hz), C<u>H</u>$_3$–C–O (3H, *s*, 1·33 ppm), two C<u>H</u>$_3$COO– (3H singlets at 1·95 and 2·27 ppm), 6 olefinic protons and 2 C=C–C<u>H</u>–OAc (8H, overlapping signals, 4·93–6·00 ppm). MS *m/e*: ($M^+$, 4), 475 ($M-AcOH^+$, 3), 444 (35), 415 (40), 397 (25), 324 (30), 306 (25), 220 (40), 105 (25), 91 (100).

*Catalytic hydrogenation.* Kodo-cytochalasin-**1** (25 mg) was hydrogenated in AcOH (10 ml) in the presence of pre-reduced $PtO_2$ (Adams) catalyst (10 mg), when after 24 hr at 25° and 715 mm, pressure absorption of $H_2$ ceased. Usual work-up gave a solid which was purified by TLC (solvent: 10% MeOH in $CHCl_3$) to furnish a product, m.p. 130–135°. MS: $M^+$, *m/e* 505.

* This behaviour is shown by some other cytochalasins, e.g. cytochalasin-D, $[\alpha]_D$ −58·9° ($CHCl_3$, *c* 0·1%), +32·6° (MeOH, *c* 0·17%).