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ISOLATION AND IDENTIFICATION OF THE METABOLIC PRODUCTS OF ASPERGILLUS PULVINUS KWON AND FENNEL COMPARATIVE STUDIES OF PRODUCTION OF TERREIN AND ERGOSTEROL IN DIFFERENT MEDIA

IZHAR H. QURESHI, TAHIRA BEGUM and RADIA NOORANI

PCSIR Laboratories, Karachi 39

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Abstract. *Aspergillus pulvinus* Kwon and Fennel, when grown on semisynthetic medium. produces terrein (I), ergosterol, stearic acid and mannitol. The metabolic products of this mold have not been described previously.

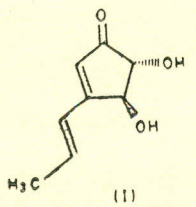
The effect of the change of medium on the yield of metabolites was also noted. Thus it was observed that the Czapek-Dox medium enhanced, the yield of terrein whereas modified Moyer and Coghill medium favoured the production of ergosterol. Terrein has been shown to have antibacterial activity.

In our continued efforts to isolate from indigenous sources, new molds capable of elaborating metabolites of pharmaceutical or economic importance, an interesting genus of Aspergillus was isolated from local soil which was identified by the Commonwealth Mycological Institute as *Aspergillus pulvinus* (Kwon and Fennel). Although the type culture was first isolated in 1962,[1] no work on its metabolic products has so far been reported in the literature. We, therefore, thought it worthwhile to undertake this study.

Earlier work from these Laboratories demonstrated the usefulness of semisynthetic media[2] (i.e. synthetic media enriched with carrot extract). Hence it was felt to use this medium in the present studies also. Thus *Aspergillus pulvinus* was grown on Czapek-Dox medium enriched with carrot extract containing 5% glucose as source of carbon and diammonium tartarate as source of nitrogen and incubated at 27° for 18 days. The mycelium was separated from the broth by filteration and then both were extracted separately.

Broth

Repeated extraction of the broth with ethyl acetate afforded a crystalline solid, m.p. 127°, $[\alpha]^{25}_{D}+135$. The compound analysed for $C_8H_{10}O_3$ was identified as terrein (I) by direct comparison with an authentic sample.



(I)

Terrein is a known metabolite of several fungi imperfecti[2-5] whose structure was established by Barton and his coworkers,[6] and which has recently been synthesised by Auerbach and Weinreb.[7]

Terrein has been shown to have antibacterial activity (Table 1). Whereas it inhibited the growth of Gram negative organisms in 0.5% concentration, a still higher concentration (1% and 1.5%) of terrein was required for inhibiting the growth of Gram positive organism like *Bacillus subtilis*, *Bacillus anthracis* and *Staphylococcus aureus*.

TABLE 1. ANTIBACTERIAL ACTIVITY OF TERREIN.

Bacteria	%Terrein*			
	0.25	0.5	1	1.5
Salmonella typhi	+	+	+	+
Salmonella paratyphi B	—	+	+	+
Klebsiella pneumoniae	—	+	+	+
Escherichia coli	—	+	+	+
Shigella sonnei	—	+	+	+
Pseudomonas aeruginosa	—	+	+	+
Staphylococcus aureus	—	—	—	+
Bacillus subtilis	—	—	+	+
Bacillus anthracis	—	—	+	+
10 *Clostridium histolyticum*	—	+	+	+

*Aqueous solution (w/v); + inhibition; —no inhibition.

Mycelium

The dried and powdered mycelium was extracted (Soxhlet apparatus) successively with petroleum ether (b.p. 60-80°), ether, ethyl acetate, chloroform and methanol.

Petroleum Ether Extract. Removal of the solvent gave an oil, which was subjected to alkaline hydrolysis,[8] Extraction of the unsaponified material with ether and removal of the solvent gave a white crystalline compound m.p. 161°, $[\alpha]^{25}_{D}$—171. It showed positive Liebermann Burchard[9] test for sterol and was identified as ergosterol by comparison with an authentic sample.

The saponified fraction on acidification and extraction with ether gave a semisolid which on crystallization from cold methanol gave a white crystalline solid, m.p. 69°. It analysed for $C_{18}H_{36}O_2$ and was identified as stearic acid confirmed by identical R_f , value[10] and undepressed mixed melting point with that of an authentic sample.

Ether, Ethyl Acetate and Chloroform Extracts. Only negligible amount of an oil was obtained which could not be purified or crystallised and, therefore, was not persued further.

Methanol Extract. Removal of solvent afforded a white crystalline solid, m.p. 166°. The compound was identified as mannitol, confirmed by identical IR spectra and undepressed mixed melting point with that of an authentic sample of mannitol. It formed hexacetate, m.p. 119° (lit m.p. 123°).

In view of the importance of terrein as antibacterial agent and ergosterol as precursor in the synthesis of vitamin D_2 it was considered worthwhile to investigate conditions for maximum production of these metabolites. In this connection, the effect of change of media on the yield of metabolites was studied.

The results (Table 2) suggest, that the production

TABLE 2

Medium	Broth (Volume (litre)	pH	Terrein)	Mycelium (Dried mycelium	Ergos-) terol
Czapek-Dox	13.6	5.5	160 mg	223 g	65 mg
Modified Moyer and Coghill	13.2	7.5	12 mg	321 g	140 mg
Raulin Thom	13.5	6.0	22 mg	100 g	23 mg
Findlays	13.8	2.5	Nil	188 g	25 mg

of terrein was maximum in Czapek Dox medium, whereas the modified Moyer and Coghill medium enhanced the yield of ergosterol.

Experimental

Melting points are recorded on Kofler block and are corrected. UV spectra were measured on a Beckmann model D.B. spectrophotometer in methanol. IR spectra were recorded on Perkin Elmer 137 spectrophotometer in KBr, unless otherwise stated. PMR spectra were taken in DMSO on Varian A60, using tetramethylsilane as internal reference.

Organism. Aspergillus pulvinus Kwon and Fennel was isolated by one of us (R.N.) from local soil and was confirmed by the Commonwealth Mycological Institute, England.

Aspergillus pulvinus was first inoculated on ordinary Czapek-Dox medium in test tubes and incubated at 27° for 9 days. This nine-day old culture was then used to inoculate flasks containing the culture medium.

Preparation of Carrot Extract. As described in ref. 2.

Cultural Conditions. The Czapek-Dox medium used was composed of glucose, 50.0 g; KH_2PO_4, 1.0 g, KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; aqueous carrot extract one litre.

In a typical batch 50 (1 litre) conical flasks, each containing 340 ml of the above medium, were autoclaved at 10 lb pressure for 20 min. Diammonium tartarate solution (75.6g litre) was sterilized and 10 ml of it was added asceptically to each of the flasks. This precaution was taken to prevent discoloration of the medium which occurs when diammonium tartarate is sterilized alongwith the medium.

These flasks containing culture medium (pH 4.5) were inoculated with 9-day old tube culture of *Aspergillus pulvinus* and incubated at 27° for 18 days till there was no more consumption of glucose as determined polarimetrically.

Isolation of Terrein (I). After 18 days the mycelium was removed by filtration and the broth (13.6 litres, pH 5.5) was acidified (pH~1) and extracted with ethyl acetate. The combined organic layer was washed with water and dried (Na_2SO_4). Removal of solvent gave a gummy product which on crystallisation from ether-petroleum ether afforded terrein (160 mg), m.p. 127 $[\alpha]^{25}_D$+135 (c, 1.2 in methanol) (Found: C, 62.29; H, 6.68%; terrein $C_8H_{10}O_3$ requires: C, 62.32; H, 6.54%; λ_{max} 271 nm (ε 25700) ν_{max} (KBr) 3620, 3420 (OH), 1701, 1639, 1570 Cm^{-1}

PMR spectrum showed signals at δ 1.78 (3H, d, J 7Hz, H_3C—CH=CH—), δ 3.92 and δ 4.5 (2×H, d, J 7.5 Hz,—*CH*OH—*CH*OH—) δ 5.72 (1H, m, H_3C—*CH*=CH—), δ 6.0 (2×H, broad singlet, —CH*OH*—CH*OH*—), δ 6.43 (IH, S, >C=*CH*—C=O), δ 6.65 (1H, d, J 8Hz,H_3C—CH=*CH*—).

Isolation of Ergosterol. The mycelium was dried overnight at 55° in an oven (wt. of dried mycelium from 50 flasks was 223), and then powdered. The powdered mycelium was extracted (Soxhlet extractor) with petroleum ether, b.p. 60-80°C for 2 days. Removal of solvent gave an oil which was saponified by refluxing with KOH (alc) 80 ml, KOH, 20g in 80% ethanol 100 ml). Extraction of the unsaponified product with ether and crystallization from ethanol gave white crystals of ergosterol, m.p. 161° (65 mg) $[\alpha]^{25}_D$—171 (0.52% in chloroform). (Found: C 81.36; H, 10.67%. Ergosterol $C_{28}H_{44}OH_2O$ requires: C, 81.10; H,11.18% λ_{max} 293, 281, 271 and 260 nm.) It gave positive Liebermann Burchard and Rosenheim tests.

Isolation of Stearic Acid The saponified fraction was acidified (pH~1) with 2N HCl and extracted with ether. Removal of solvent gave a waxy solid which on crystallisation from cold aqueous methanol furnished stearic acid, m.p. 69° (5.4 g). (Found, C.76.23; H, 12.4%. Stearic acid, $C_{18}H_{36}O_2$, requires: C, 76.00; H, 12.76%), ν_{max} 2924, 2874, 1709, 1439, 1414, 1302, 940 cm^{-1}.

Isolation of Mannitol. The methanol extract of mycelium on removal of solvent and crystallization of the product with methanol gave mannitol, m.p. 166° (9.0g). It formed hexaacetate, m.p. 119°. (lit. m.p. 123°).

To study the effect of change of medium on the yield of terrein (I) and ergosterol, *Aspergillus pulvinus* was also grown in the following media enriched with carrot extract: (a) modified Moyer and Coghill medium[11] (molasses was used in place of lactose); (b) Raulin-Thom medium;[12] and (c) Findlay's medium.[13]

The results are summarised in Table 2.

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