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Part I. Isolation and Characterization of Gliotoxin, Ergosterol, Palmitic Acid and Mannitol—Metabolic Products of Trichoderma Hamatum Bainier

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Abstract. The metabolic products of *Trichoderma hamatum* Bainier are described for the first time. It has been shown that this mould, when grown on a semisynthetic medium, produces the compounds mentioned in the title.

During screening of fungi from indigenous sources a strain of *Trichoderma* was isolated which was identified[1] as *Trichoderma hamatum* Bainier. A survey of the literature showed that although this mould was first described[2] in 1913, its metabolic products have not been investigated so far. We, therefore, undertook this study.

Trichoderma hamatum Bainier was grown on Findlay's medium enriched with carrot extract[3] and incubated at 24°C for 16 days. The mycelium was separated from the broth by filtration and then both were worked up separately.

Broth

The ethyl acetate extract of the broth, on being taken to dryness, furnished a brown viscous residue which on extraction with benzene and removal of solvent yielded a brown solid (m.p. 184–189°C). Preparative TLC (silica gel) resolved it into five compounds (Table 1).

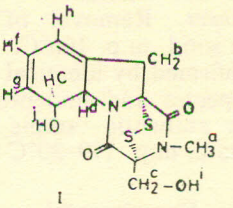
The results indicate that compound C, was the major component (*ca.* 50% of the crude mixture) and hence its characterization was undertaken. Other fractions, being obtained in small quantities, could not be pursued further.

Identification of Compound C. It had m.p. 220–21°C (dec), $[\alpha]_D^{25}$—250. Its elemental analysis coupled with mass measurement (M+326) suggested to have the molecular formula, $C_{13}H_{14}O_4N_2S_2$.

Its IR spectrum (in KBr) showed absorptions at 3344 (—OH), 1695 (>C=O), 1667 (>C=C<) and 1439 (—CH_3) cm^{-1}. In UV it absorbed at λ_{max} 265 nm (ε 4490).

The PMR spectrum (in DMSO) showed a singlet at δ 3.05 (—CH_3, a) for a methyl group. This low δ value (3.05) suggested that this methyl group was attached to a heteroatom. A broad hump at δ 3.23 (—*OH*, i) and another hump at δ 3.50 (—OH, j) could be ascribed to the two alcoholic protons. A doublet at δ 4.2 (—CH_2, c; *J* 2 Hz) and a singlet at δ 4.3 (—CH_2, b) were due to two methylene groups. An ill defined doublet at δ 4.59 (>C(—H)—, d, *J* 2 Hz) and a multiplet at δ 4.67 (>C(—H)—, e) were indicative of two methine protons. Signals at δ 5.67, δ 5.9 and δ 6.0 were compatible with the three olefinic protons (h, f and g).

On the basis of above analytical and spectral data, compound C was identified as Gliotoxin (I).



I

Gliotoxin is known to be a highly antifungal and antibacterial metabolite of several *Fungi imperfecti*.[4] It was first isolatedd in 1936 by Weindling[5] from *Gliocladium fimbriatum* and later on by Brain[6] from *Trichoderma viridi* and Raistrick[7] from *Penicillium cinerasceus*. The chemistry of gliotoxin was extensively studied by Johnson and his coworkers,[8] while its correct structure was proposed by Woodward[9] in 1958 and later on confirmed by X-ray analysis.[10]

Mycelium

The dried mycelium was extracted (Soxhlet apparatus) with petroleum-ether, benzene, ethyl acetate, chloroform and ethanol respectively.

TABLE 1.

Compound	R_f	M.p. (°C)	$[\alpha]_D^{25}$	Yield mg/litre of broth
A	0·00	124–125	-52·77	2·3
B	0·17	160–162	-144·9	1·2
C	0·28	220–221 (dec)	-250	216·3
D	0·53	230 (dec)	-12·3	1·7
E	0·98	240 (dec)	-53·3	0·8

Petroleum-ether Extract. Removal of solvent gave an oily material which was subjected to alkaline hydrolysis. Extraction of the unsaponified fraction with ether gave a white solid which on crystallisation from methanol had m.p. 167–8°C, $[\alpha]_D^{25}$—170. Its UV spectrum showed bands at λ_{max} 292, 281, 270 and 260 nm characteristic for ergosterol.

The compound was identified as ergosterol, confirmed by identical *Rf* values, and undepressed mixed m.p. with that of an authentic sample of ergosterol.

The saponified fraction on acidification and extraction with ether gave a waxy solid which on crystallisation from dilute methanol afforded white crystals, m.p. 62–63°C. It was identified as palmitic acid, confirmed by identical *Rf* values, superimposable IR spectra and undepressed mixed m.p. with that of authentic sample of palmitic acid. It formed amide, m.p. 106°C (lit. m.p. 106°C).

Benzene, Ethyl Acetate and Chloroform Extracts. Removal of solvent from each extract gave negligible amounts of oily material which could not be purified and hence were not studied.

Ethanol Extract. Removal of solvent yielded white crystalline solid, m.p. 166°C. It was identified as mannitol, confirmed by identical *Rf* values, superimposable IR spectra and undepressed mixed m.p. with that of an authentic sample of mannitol. It formed hexaacetate, m.p. 119–20°C (lit. m.p. 121°C)

Experimental

Melting points were taken on a Kofler block and are corrected. UV spectra were determined in ethanol (95%) on Beckman DB spectrophotometer. IR spectrum were recorded on a Perkin–Elmer 137 instrument in KBr, unless otherwise stated. PMR spectra were measured on Varian A–60 using TMS as internal reference. Mass spectra were measured on AEI MS 9 at 70 eV. Petroleum ether used had b.p. 60–80°C. Microanalyses were carried out by Dr. F.B. Strauss, Microanalytical Laboratory, Oxford, England.

Organism. During screening for new strains and from indigenous sources, a strain of *Trichoderma* was isolated by one of us (R.N.). It was later on identified by C.M.I. as *Trichoderma hamatum* Bainier (IMI–139407).

Trichoderma hamatum was first grown an Czapeck-Dox medium in test tubes, and incubated at 24°C for 9 days. This 9 day-old culture was then used to inoculate flasks containing the culture medium.

Cultural Conditions. The modified Findlay's medium used was composed of : glucose 32.7, NH_4Cl 1.2; $KH_2PO_4.7H_2O$, 2.5; $MgSO_4.7H_2O$, 1.0; $FeSO_4.7H_2O$, 0.01; $ZnSO_4$, 0.01; $CuSO_4.7H_2O$, 0.005; sodium borate, 0.005; vit B, 0.005; and sodium acetate, 1 g.

These ingredients were added to aqueous carrot extract, prepared from 200 g peeled carrots[3] and the volume was made up to one litre.

In a typical batch, 15 conical flasks (1-litre) were taken, each containing 350 ml of the above medium (pH 5.0) and autoclaved at 10 lb pressure for 20 min. These flasks containing culture medium were then inoculated with 9–day old tube culture of *Trichoderma hamatum* and incubated at 24°C, for 16 days.

TABLE 2.

Day of incubation	α	Glucose(%)
1st	2·42	5·1
8th	2·16	4·2
10th	1·57	3·0
12th	1·42	2·70
14th	1·1	2·09
16th	1·0	1·90
17th	1·0	1·90

During incubation, broth (10 ml) was drawn asceptically, first after a week and then every second day for optical rotation measurement. The data (Table 2) indicated that after 16 days glucose ceased to be metabolised.

Broth

After 16 days, mycelium was filtered off and the broth (5.25 litre, pH 2.2) was extracted with ethyl acetate. The ethyl acetate extract was dried Na_2SO_4 and the solvent was removed *in vacuo* leaving a gummy product. Extraction of this gummy material with benzene, afforded a solid m.p. 184–89°C (2.44 g) which on purification through preparative TLC (0.5 mm, 20×20 cm, silica gel plates using chloform as mobile phase) gave gliotoxin (1.2 g), *Rf* 0.28. On recrystallization from methanol gliotoxin had m.p. 220–221°C, $[\alpha]_D^{25}$—250 (*C*, 0.114 in chloroform) [lit m. p. 221°C, $[\alpha]_D^{25}$—255 (*C*, 0.103 in chloroform) Found: C 47.83%, H4.33%, N 8.57%, and S 19.68%. Gliotoxin $C_{13}H_{14}N_2S_2O_4$; requires: C 47.84, H 4.32, N8.54 and S 19.65%. λ_{max} 265 (ε 4410) nm μ_{max} 3344, 1695, 1667, 1437 cm^{-1}. PMR (DMSO) δ 3.05 (3 Ha, singlet), δ 3.23 (OHi, hump), δ 3.50 (OHj, hump), δ 4.20 (2Hc, doublet *J* 2Hz), δ 4.30 (2Hb, singlet) δ 4.59 (1Hd, doublet *J* 2Hz), δ 4.67 (1He, multiplet), δ 5.67, δ 5.9 and δ 6.0 (olefenic protons h, f and g); Mass (Found: *m/e* 326.044 $C_{13}H_{14}O_4N_2S_2$ requires: 326.032). Base peak was formed at *m/e* 262 directly from the molecular ionic peak by the loss of 64 (—2×S) mass units. Other intense peaks present in the spectrum were *m/e* 244, 233, 217, 216, 215, 205, 203, 201, 189, 160, 144, 143, 141, 135, 134, 131, 117, 116 and 115.

Mycelium

The mycelium was dried overnight in an oven at 60°C. The dried mycelium (105 g from 15 flasks) was powdered and extracted (Soxhlet apparatus) with petroleum-ether, benzene, ethyl acetate, chloroform and ethanol respectively.

Petroleum-ether Extract. Removal of the solvent furnished an oily material (2.02 g) which was subjected to alkaline hydrolysis.

Isolation of Ergosterol. Oily material (1 g), in 10 ml 20% alcoholic KOH was taken up in a

round-bottom flask fitted with a condenser carrying soda lime tube and was refluxed on steam bath for 4 hr. The unsaponified fraction was extracted with ether. Removal of solvent followed by crystallisation with ether afforded ergosterol as colourless needles, m. p. 167–168°C (14.5 mg), $[\alpha]_D^{25}$ — 170. It gave a positive Rosenheim and Libermann–Burchard tests; λ_{max} 292, 281, 270 and 260 nm.

Isolation of Palmitic Acid. The saponified fraction was acidified with 2N HCl and was thoroughly extracted with ether. The combined organic layer was washed with water, dried (Na_2SO_4) and the solvent was distilled off. Crystallization from dil. methanol gave palmitic acid as crystalline needles, m.p. 62–3°C (lit. m.p. 63–4°C) ν_{max} 2941, 2857, 1965, 1312, 1292, 1267, 1250, 1227, 1205 and 1190 cm^{-1}. Treatment of palmitic acid (10 mg) with thionyl chloride (2 ml) and then reacting the resulting acid chloride with ammonia (excess) yielded palmitamide, m.p. 106°C (lit. m.p. 106°C).

Isolation of Mannitol. The ethanol extract on removal of solvent yielded mannitol as white crystals, m.p. 166°C (105 mg). It gave a positive boric acid test for polyhydric alcohols ν_{max} 3356, 2985, 1460, 1343, 1379, 1361, 1328, 1266, 1198, 1086, 1029, 955, 925, 885 cm^{-1}. It formed hexaacetate, m.p. 119–120°C (lit. m.p. 120–121°C).

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