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THE ISOLATION AND IDENTIFICATION OF A NEW METABOLITE FROM ASPERGILLUS FUMIGATUS RELATED TO GLIOTOXIN

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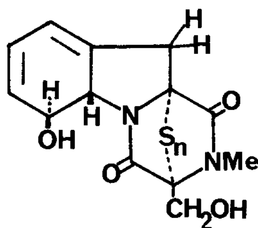
Summary: Gliotoxin G, the tetrasulphide analogue of gliotoxin has been characterized from a laboratory isolate of Aspergillus fumigatus.



I

Gliotoxin (I, n = 2) belongs to the class of fungal metabolites possessing the epidithiodioxopiperazine ring structure.[1] These compounds possess antifungal, antiviral and antimicrobial properties in vitro but their toxicity has precluded any therapeutic use. Gliotoxin was originally isolated from Gliocladium fimbriatum[2] and has been isolated from Aspergillus fumigatus[3] and Penicillium terlikowskii[4]. Recently, gliotoxin was shown to have potent immunomodulating activity[5] when tested in mammalian immune systems. During the course of this work a second metabolite was detected by thin layer chromatography when A. fumigatus was cultured in Eagles minimum essential media F15[6] (Grand Island Biologicals, Grand Island, NY). This compound also possessed biological activity. We now report the isolation and identification of this compound.

The laboratory isolate of A fumigatus[7] was grown in F15 media at 24° as previously described in reference 6. The mycelium was separated from the supernatant (1l) by filtration first through cheesecloth followed by Whatmans No4 paper. Extraction with chloroform (3 x 300 mls), drying over calcium sulphate and evaporation under vacuum gave a dark oil (50 mg). Examination on

thin layer chromatography plates (Merck 5 x 10 cm silica plates $60F_{254}$ in 5% methanol in dichloromethane) showed two principal components, gliotoxin (Rf 0.50) and a second band at Rf 0.34. The latter was removed from the plate and the crushed silica extracted with acetone (3 x 50 mls). Evaporation, drying and redissolution in chloroform to remove trace silica followed by a second evaporation gave an oil (5 mg). This material, although apparently homogeneous by tlc in two solvent systems (ethylacetate, Rf 0.5 and 5% methanol in dichloromethane) was resolved into two components by multiple developments of silica tlc plates in diethylether. The two components had Rf 0.22(A) and 0.26(B) after three developments. Preparative thin layer chromatography (Merck 20 x 20 x 1 mm $60F_{254}$ silica gel) in ether gave A(~1mg mp 160-165°) and B(~2 mg as an oil) as chromatographically homogeneous compounds which could not be successfully recrystallized.

Compound A NMR spectrum δ(270MHz $CDCl_3$): 3.03, 3.09, 3.22, 3.29 (2H, d of d J 16.0 Hz, benzylic H); 3.13 (3H, s, N-CH_3); 4.06, 4.11, 4.34, 4.38 (2H, d of d J 13.2 Hz, CH_2OH); 4.75, 4.80, 5.04, 5.09 (2H, d of d J 12.1 Hz, allylic H); 5.76-5.94 (3H, complex multiplet, vinylic H).

Mass spectrum 1. Electron impact gave M/e at 262, 244, 226 and 214. No fragments with M/e greater than 262 were observed.

2. Chemical ionization gave (M/e + 1) at 391, 359 (M/e + 1 - S), 327 (M/e + 1 - S_2), 263 (M/e + 1 - S_4), 245 (M/e + 1 - S_4 - H_2O), 227 (M/e + 1 - S_4 - $2H_2O$). High resolution chemical ionization gave (M/e + 1): 390.9914, expected for $C_{13}H_{15}N_2O_4S_4$ 390.9915; (M/e + 1 - S): 359.0193, expected for $C_{13}H_{15}N_2O_4S_3$ 359.0194; and (M/e + 1 - S_2): 327.0472, expected for $C_{13}H_{15}N_2O_4S_2$, 327.0473.

Optical Rotation ($CHCl_3$ conc. = 2.33 x 10^{-4} M) $[M]_{404}$ - 1890°, $[M]_{435}$ - 1,500°, $[M]_{500}$ - 970°, $[M]_{577}$ - 620°. The literature value[2] for gliotoxin is $[M]_{589}$ -840° (C = 0.103). The corresponding values for gliotoxin in our hands were $[M]_{404}$ - 1480°, $[M]_{435}$ - 1220°, $[M]_{500}$ - 864°, $[M]_{577}$ - 593° (conc. = 1.18 x 10^{-3} M).

The nmr spectrum of compound A is qualitatively the same as gliotoxin.[8] The major difference is the accidental equivalence of the allylic protons on the hydrated benzene ring in gliotoxin when this compound was run in chloroform. In DMSO (d_6) these protons in gliotoxin are seen as a doublet of doublets with a coupling of 13.0 Hz[9].

The coupling between these protons in compound A is consistent with the trans diaxial orientation as in gliotoxin[10]. The electron impact mass spectrum of

compound A strongly suggests a molecule closely related to gliotoxin since the observed fragmentation patterns below M/e 262 are the same in both compounds when run under identical conditions suggesting a common fragment at 262 which does not contain sulphur. The M/e fragment at 262 is the highest mass seen in the normal electron impact mass spectrum of gliotoxin and corresponds to the gliotoxin - S_2 fragment[11]. However, the chemical ionization mass spectrum revealed, as well as the protonated dithio fragment at 327, peaks due to an S_3 and S_4 compound. The high resolution mass measurements confirmed this. On the basis of this evidence compound A has been assigned the structure I, n = 4 and named gliotoxin-G by analogy with sporidesmin G, the S_4 derivative of sporidesmin[12]. The ORD curve for gliotoxin G between 400 and 577 nm is very similar to that of authentic gliotoxin except for enhancement of 15-30% suggesting the same absolute configuration of the disulphide.

Compound B NMR spectrum δ ($CDCl_3$) 2.25 (3H, s, S-CH_3); 2.27 (3H, s, S-CH_3); 2.90, 2.96, 3.06, 3.13 (2H, d of d J 16.2 Hz, benzylic H); 3.15 (3H, s, N-CH_3); 3.90, 3.95, 4.37, 4.41 (2H, d of d J 12.0 Hz, CH_2OH); 4.93 (2H, br.s, allylic H). 5.75 - 5.98 (3H, complex multiplet, vinylic H).

Electron impact mass spectrum M/e 356, 309, 262, 244. High resolution mass spectrum gave M/e: 356.0862 expected for $C_{15}H_{20}N_2O_4S_2$, 356.0865 and M/e: 309.0904 expected for $C_{14}H_{17}N_2O_4S$: 309.0861. (M/e - SCH_3)
The nmr spectrum of this compound is identical to the dethiobisdithiomethylether derivative of gliotoxin isolated by Kirby et al[13] from Gliocladium deliquescens. The elemental composition derived from high resolution mass measurements confirms this.

Gliotoxin G is biologically active when tested using the macrophage cell adherence to plastic assay[5]. The dithiomethylether is inactive. The biological properties of both of these compounds are under investigation.

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6. Mullbacher, A., Waring, P. and Eichner, R.D. J.Gen.Microbiol., 131, 1251-1258, 1985. F15 is a complex media routinely used for tissue culture of mammalian cells. It is noteworthy that the strain of *A. fumigatus* investigated did not produce the tetrasulphide when grown in Czapok Dox or corn steep liquor media. In our hands *P. terlikowskii* in Weindling media produced mainly gliotoxin and the dethiobisdithiomethylether of gliotoxin but a small quantity of tetrasulphide (<5%) was detected. It could not be freed from the much larger quantities of thiomethylether produced by this organism.

7. The organism was kindly identified by Dr. Dorothy Frey, Australian National Reference Laboratory in Medical Mycology, Royal Northshore Hospital, NSW 2065, Australia.

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9. The nmr of gliotoxin in DMSO (d_6) had δ(ppm) : 2.98, 3.16, 3.72, 3.55 (2H, d of d, J 16.0 Hz, benzylic), 3.10(3H,s,N-CH_3), 4.13, 4.27, 4.30 4.44 (2H, d of d, J 12.0 Hz CH_2OH), 4.43, 4.62, 4.81, 4.95 (2H, d of d, J 13.0 Hz, allylic H) 5.71-6.04 (3H, multiplet, vinylic H). In DMSO the methylene protons of the primary alcohol fragment appear as a tightly coupled AB quartet. Assignment of these signals in DMSO followed from a temperature study at 30°, 50°, 70° and 90°. The signal centred on 4.28 ppm collapsed to a broad singlet with progressive change in peak width at half height from 19.3 Hz to 11.0 Hz on heating from 30° to 90°. The quartet centred on 4.72 ppm was unaffected. The conformationally mobile methylene side chain would be expected to show a sharper signal with more rapid rotation about the C-C bond.

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