Biosynthesis of Bisdethiobis(methylthio)gliotoxin, a New Metabolite of *Gliocladium deliquescens*

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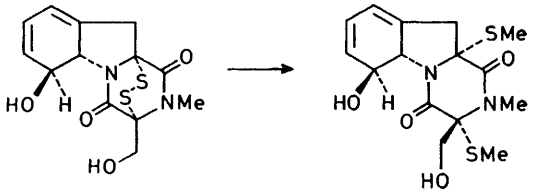
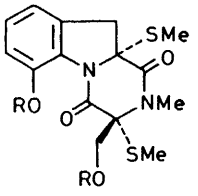
Bisdethiobis(methylthio)gliotoxin (2) has been identified as an amorphous, minor metabolite of *Gliocladium deliquescens* and characterised *via* the crystalline bis-4-bromobenzoate of the corresponding didehydro-derivative. The new metabolite has been synthesised from gliotoxin (1) by reduction and methylation. Feeding experiments with [^{14}C]-(1) and -(2), prepared biosynthetically from L-[*U*-^{14}C]phenylalanine, have shown that bisdethiobis-(methylthio)gliotoxin is formed (8.6% incorporation), apparently irreversibly, from gliotoxin in *G. deliquescens*.

DURING studies [1] into the biosynthesis of gliotoxin (1), the major sulphur-containing metabolite of *Gliocladium deliquescens*, we carried out control feeding experiments with L-[*U*-^{14}C]phenylalanine. The resulting mixture of metabolites was examined for radioactive products by autoradiography of t.l.c. plates. At least eight radioactive components were seen to accompany gliotoxin. We selected the most prominent component for further study and now report on the structure, partial synthesis, and biosynthesis of this new metabolite, bisdethiobis-(methylthio)gliotoxin (2).



(1) (2)



(3)

Gliocladium deliquescens was grown under the usual conditions for four days. The culture filtrate was extracted with ethyl acetate to yield a gliotoxin-rich mixture which was separated by layer chromatography. The metabolite (2) was obtained, in *ca.* 6% of the yield of gliotoxin, as an amorphous solid which resisted crystallisation; *O*-acyl derivatives of (2) were likewise not obtained crystalline. However, the structure (2) was readily discerned by n.m.r. spectroscopy and established unambiguously by partial synthesis from gliotoxin. Thus, reduction and methylation of gliotoxin (1), under conditions used earlier [2] with sporidesmin, gave, in good yield, the derivative (2) which was judged chromatographically and spectroscopically to be identical with the natural material. Again, the partially synthetic sample of (2) did not crystallise, although satisfactory analytical data were obtained with the amorphous material. Nevertheless, a crystalline derivative was sought to facilitate the biosynthetic studies described in the sequel. Dehydrogenation of gliotoxin (1) with tetrachloro-1,2-benzoquinone in benzene, to give the corresponding phenol, has been reported earlier.[3] Similarly, we found that treatment of (2) with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in chloroform gave the phenol (3; R = H) in good yield. This amorphous product formed a crystalline bis-4-bromobenzoate (3; R = 4-BrC_6H_4CO) which was fully characterised. Samples of (3; R = 4-BrC_6H_4CO) prepared from natural and partially synthetic (2) were identical.

The biosynthesis of the metabolite (2) in *Gliocladium deliquescens* was investigated with [^{14}C]-labelled precursors. In preliminary experiments, Dr. G. Bianchi monitored the uptake of radioactivity from L-[*U*-^{14}C]phenylalanine into the metabolites of *G. deliquescens* by the radioscanning of t.l.c. plates. Substantial amounts of ^{14}C appeared in gliotoxin within 1 h of addition of labelled phenylalanine to the culture medium, but much longer incubation times ($\geqslant$6 h) were required before a radioactive band corresponding to (2) could be detected confidently. This suggested, although it did not prove, that gliotoxin (1) was converted biochemically into (2) in a manner analogous to the chemical conversion described above. The possible biosynthetic interconversions, (1) $\rightleftharpoons$ (2), were tested directly as follows.

[^{14}C]Gliotoxin was prepared biosynthetically from L-[*U*-^{14}C]phenylalanine by incubation with *Gliocladium deliquescens*. Separation of [^{14}C]gliotoxin from the co-occurring labelled metabolite (2) was effected by layer chromatography and the gliotoxin was purified further by crystallisation to constant specific activity. The [^{14}C]gliotoxin was fed in dimethyl sulphoxide to cultures of *G. deliquescens* and, after three days, the resulting metabolites were isolated and diluted with non-radioactive (2) prepared chemically from (1). The mixture was separated chromatographically to give gliotoxin (1) and the metabolite (2). The gliotoxin, after repeated crystallisation, had a specific activity corresponding to a 27.4% recovery of the activity fed. The metabolite (2) was converted into (3; R = 4-BrC_6H_4CO) which, after crystallisation, had an activity corresponding to an 8.6% incorporation of gliotoxin. In a duplicate

experiment, recovery of gliotoxin (26.1%) and incorporation into (2) (8.6%) were observed.

A complementary experiment was carried out to test the possibility that (2) might be demethylated and oxidised *in vivo* to reform (1). Accordingly, [^{14}C]-(2) was prepared from [^{14}C]-(1) and fed in dimethyl sulphoxide to *Gliocladium deliquescens*. The resulting mixture of metabolites (58% recovery of activity) was examined by the radioscanning of t.l.c. plates; essentially all the radioactivity was associated with (2). Moreover, the gliotoxin was found, after recrystallisation, to be inactive [<0.2% incorporation of (2)].

We conclude that the new, minor metabolite of *Gliocladium deliquescens*, bisdethiobis(methylthio)gliotoxin (2), is formed in the fungus by irreversible reduction and methylation of gliotoxin (1). Other bis(methylthio)-derivatives, found in various fungi,[4] may arise similarly from recognised epidisulphides or through the 'trapping', by methylation, of transient dithiol intermediates on the biosynthetic pathways.

EXPERIMENTAL

General.—M.p.s were measured with a Kofler hot-stage apparatus. I.r. spectra were recorded for KBr discs, u.v. spectra for ethanol solutions, and n.m.r. spectra for $CDCl_3$ solutions at 90 MHz. Layer chromatography was carried out generally on silica F_{254} plates of 0.75 or 0.25 mm thickness developed with benzene–acetone (3 : 1); typical R_F values were, gliotoxin (1) 0.40, bisdethiobis(methylthio)-gliotoxin (2) 0.25, and viridiol 0.20.

Radiochemical Methods.—^{14}C Activities were measured with a Philips liquid scintillation analyser using toluene–methanol solutions. A Panax thin layer scanner RTLS-1A was used for the radioscanning of t.l.c. plates.

Conversion of Gliotoxin (1) *into Bisdethiobis(methylthio)-gliotoxin* (2).—Iodomethane (5 ml) was added at 0° to a solution of gliotoxin (326 mg) in pyridine (1 ml). The resulting yellow precipitate was dissolved by addition of methanol (2 ml). Sodium borohydride (100 mg) in methanol (2 ml) and iodomethane (2 ml) were added successively and the mixture stirred at room temperature for 4 h. The solution was evaporated and the residue partitioned between water (25 ml) and chloroform (3 × 50 ml). Evaporation of the chloroform extract gave a gum (328 mg) judged by t.l.c. and n.m.r. spectroscopy to be largely (2). Preparative t.l.c. gave *bisdethiobis(methylthio)-gliotoxin* (2) as a foam (Found: C, 50.8; H, 5.75; N, 7.6. $C_{15}H_{20}N_2O_4S_2$ requires C, 50.8; H, 5.6; N, 7.9%), δ 2.20 (s, SMe), 2.23 (s, SMe), 2.99br (s, allylic CH_2), 3.11 (s, NMe), 3.88 and 4.38 (ABq, *J* 12 Hz, OCH_2), 4.90br (s, 2 H, methine H), and 5.6—6.0 (m, 3 H, vinyl H).

Dehydrogenation of Bisdethiobis(methylthio)gliotoxin (2).—The sulphide (2) (300 mg) was heated under reflux with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (250 mg) in chloroform (30 ml) for 6 h. The mixture was filtered and the filtrate evaporated. The residue was purified by preparative t.l.c. to give didehydrobisdethiobis(methylthio)-gliotoxin (3; R = H) (204 mg) as a gum, δ 2.20 (s, SMe), 2.30 (s, SMe), 2.67 (m, disappeared upon addition of D_2O, alcoholic OH), 3.18 (s, NMe), 3.44 and 3.55 (ABq, *J* 17 Hz, benzylic CH_2), 3.96 and 4.53br (ABq, sharpened upon addition of D_2O, *J* 12 Hz, OCH_2), 6.7—7.2 (m, aryl H), and 10.19 [s, disappeared upon addition of D_2O, phenolic OH]. The *bis-4-bromobenzoate* (3; R = 4-BrC_6H_4CO), prepared using 4-bromobenzoyl chloride in pyridine and purified by t.l.c. (silica plates; benzene–acetone 9 : 1), formed prisms (from Et_2O–MeOH), m.p. 150—151°, $[\alpha]_D$ −160° (*c* 0.7 in $CHCl_3$) (Found: C, 48.6; H, 3.4; Br, 22.3; N, 3.7; S, 8.7. $C_{29}H_{24}Br_2N_2O_6S_2$ requires C, 48.4; H, 3.4; Br, 22.3; N, 3.9; S, 8.9%), $\lambda_{max.}$ 246 nm (ε 6 900); $\nu_{max.}$ 1 740 and 1 680 cm^{-1}, δ 2.00 (s, SMe), 2.29 (s, SMe), 3.13 (s, NMe), 3.52 (s, benzylic CH_2), 4.63 and 4.88 (ABq, *J* 12 Hz, OCH_2), and 7.1—8.2 (m, aryl H).

Isolation of Bisdethiobis(methylthio)gliotoxin (2) *from* Gliocladium deliquescens.—*Gliocladium deliquescens* (Trichoderma viride, see ref. 1) (strain no. 1 828 NRRL), maintained on potato dextrose agar, was grown in shake-culture at pH 3.0—3.5 in a defined [5] medium (6 l) at 28—30° for 4 days. The culture filtrate was extracted with ethyl acetate (4 × 1 l). The extracts were washed with water (2 × 1 l), then brine (2 × 1 l), dried (Na_2SO_4), and evaporated to give a partially crystalline residue (900 mg). Crystallisation from chloroform–methanol gave gliotoxin (1) (500 mg). The mother-liquors were separated by t.l.c. to give, *inter alia*, bisdethiobis(methylthio)gliotoxin (2) (30 mg) which was indistinguishable by t.l.c. and n.m.r. spectroscopy from partially synthetic material (as above). The derived bis-4-bromobenzoate (3; R = 4-BrC_6H_4CO) had m.p. 150—151°, undepressed on admixture of the sample prepared from gliotoxin.

Biosynthetic Preparation of [^{14}C]*-Gliotoxin and -Bis dethiobis(methylthio)gliotoxin.*—L-[*U*-^{14}C]Phenylalanine (100 μCi, 522 mCi mmol^{-1}) was added to 1 day old cultures (4 × 50 ml) of *Gliocladium deliquescens*. After 3 days, extraction of the culture filtrate with ethyl acetate gave a mixture of metabolites (83 mg; 7.32 μCi). Preparative t.l.c. gave [^{14}C]gliotoxin (37.6 mg) which was crystallised to constant specific activity (0.10 μCi mg^{-1}). Radioscanning of a t.l.c. plate of the crystallised gliotoxin showed no contamination by the co-metabolite (2).

The [^{14}C]gliotoxin (5.9 mg; 0.10 μCi mg^{-1}) was diluted with non-radioactive gliotoxin (23.5 mg) and converted, as before, into [^{14}C]-(2) (30 mg). The product was mixed with non-radioactive gliotoxin (12 mg) and the mixture separated by t.l.c. to remove any traces of unchanged [^{14}C]gliotoxin. In this way, [^{14}C]bisdethiobis(methylthio)gliotoxin (11.7 mg, 0.018 5 μCi mg^{-1}) was obtained.

Feeding Experiments with Gliocladium deliquescens.—(a) *With* [^{14}C]*gliotoxin.* [^{14}C]Gliotoxin (5.18 mg; 0.10 μCi mg^{-1}) (prepared as before) in dimethyl sulhpoxide (1 ml) was added to 1 day old cultures (20 × 50 ml) of *G. deliquescens*. After 3 days, extraction of the culture filtrate gave a mixture of metabolites (198 mg, 45% recovery of ^{14}C). This was diluted with non-radioactive bisdethiobis(methylthio)gliotoxin (2) (146 mg) and separated by t.l.c. to yield (2) (108 mg) and gliotoxin (1) (79 mg). The gliotoxin was crystallised ($CHCl_3$–MeOH) to a constant specific activity corresponding to 27.4% recovery of the ^{14}C fed. The metabolite (2) was converted into the bis-4-bromobenzoate (3; R = 4-BrC_6H_4CO) (56 mg) which, likewise, was crystallised (Et_2O–MeOH) to constant activity (8.6% incorporation of gliotoxin). In a duplicate experiment, [^{14}C]gliotoxin (5.18 mg) in dimethyl sulphoxide (0.25 ml), fed to 1 day old cultures (5 × 50 ml), gave gliotoxin (26.1% recovered ^{14}C) and the derivative (3; R = 4-BrC_6H_4CO) (8.6% incorporation).

(b) *With* [^{14}C]*bisdethiobis(methylthio)gliotoxin.* [^{14}C]Bisdethiobis(methylthio)gliotoxin (1.05 mg; 0.018 5 μCi mg^{-1}),

prepared from [^{14}C]gliotoxin as before, in dimethyl sulphoxide (0.25 ml) was added to 1 day old cultures (5 × 50 ml) of *G. deliquescens*. After 3 days, the metabolite mixture (32 mg, 58% recovery of ^{14}C) yielded gliotoxin which lost essentially all radioactivity upon recrystallisation (<0.2% incorporation of ^{14}C).

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