BIOSYNTHESIS OF GLIOVIRIN: INCORPORATION OF L-PHENYLALANINE (1-^{13}C)

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Gliovirin[1] (**1**) and the related antibiotic, gliotoxin[2] (**2**), are produced by P- and Q-strains, respectively, of the fungus *Gliocladium virens*[3]. *G. virens* is an effective biocontrol agent of several seedling and soilborne plant diseases[4~6], and gliovirin and gliotoxin have been linked to *G. virens* efficacy as a biocontrol agent of seedling diseases incited by *Pythium ultimum* and *Rhizoctonia solani*, respectively[3]. The biosynthesis of gliotoxin has been extensively investigated, but that of gliovirin has not. During the isolation of gliovirin, L,L-phenylalanine anhydride was also isolated[1]. Based on this observation, we hypothesized that L-phenylalanine was the biosynthetic precursor of gliovirin. We now confirm this hypothesis.

Materials and Methods

Preparation of Hemicellulose

Hemicellulose was prepared using a variation of the general procedures of SELVENDRAN and RYDEN[7]. Ground millet (50 g), that passed through a 40 mesh screen after ball milling, was extracted 2× with a solution of aq 4 M KOH and 3% H_3BO_3 and the resulting extraction mixture that contained both solubles and particulates was centrifuged. The resulting pellet was suspended in water, centrifuged a second time, and washed in dialysis tubing with water for 4 days with several changes of water. The contents remaining within the tubing were sieved through a 30 mesh screen to separate fines (5.24 g) from particulates (2.67 g) (yields after freeze drying). The fines, which provided a medium that gave higher yields of gliovirin than the particulates, were used for all tests.

Fermentation

A medium composed of 0.15% NH_4NO_3, 0.025% $MgSO_4$, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.001% Fe-Na-EDTA, 1.0% 2-[*N*-morpholine]ethanesulfonic acid (MES) buffer, 0.2% hemicellulose, and 0.2% of the appropriate amino acid in 1 liter of distilled water was prepared. The pH was adjusted to 6.1 with 2 N HCl. Conidia of *G. Virens* strain G-4 were washed from 7-day-old Potato Dextrose agar plates with sterile distilled H_2O. Aliquants of conidia suspension (1 ml) were added to 125-ml Erlenmeyer flasks containing 50 ml of the above medium to make a final concentration of 1×10^5 conidia ml^{-1}. The inoculated flasks were incubated on a rotary shaker at 24°C and 150 rpm for 5 days.

Isolation and Purification

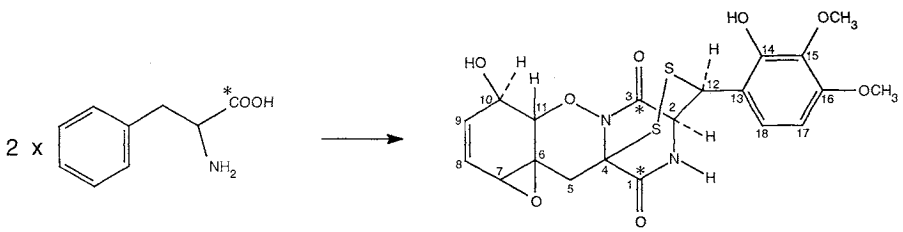
The cultures were centrifuged and the pellet was extracted with 80% aqueous acetone. The acetone was removed *in vacuo* and the residue was added to the supernatant. This mixture was extracted with an equal volume of EtOAc. After concentrating the extract *in vacuo*, the residue was dissolved in 1 ml of methanol and subjected to HPLC analysis or streaked on TLC plates of Silicar 4GF (Mallinckrodt Chemical Works). The plates were developed in chloroform-acetone (70:30) and observed under 250 nm UV light. A dark absorbing zone at Rf 0.30 was eluted with chloroform-methanol (50:50) (solvent 1), streaked on another TLC plate, and developed with EtOAc. A dark absorbing zone at Rf 0.59 was eluted with solvent 1. The product was subjected to ^{13}C and/or ^{1}H NMR and HPLC analysis.

HPLC Analysis

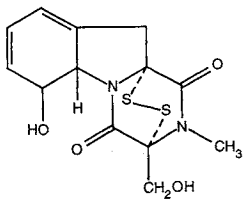
The extracts were chromatographed on a C-18 column (Scientific Glass Engineering, 250 × 4.6 mm, 5 μm packing) at 40°C. The mobile phase (1.25 ml/minute) consisted of dilute acid (0.07% H_3PO_4) and acetonitrile (ACN). Development of the chromatogram was isocratic (80% acid: 20% ACN) for 5 minutes, followed by a linear gradient to 72% acid: 28% ACN over 1 minute, and held at this ratio for an additional 12 minutes. The eluent was monitored at 254 mm with a diode array detector. The instrument was calibrated by injecting known quantities of authentic gliovirin.

Results and Discussion

G. virens (strain G-4) readily synthesizes gliovirin



(1)



(2)

(2,370 μg/ml) when grown on a millet medium as shown by HPLC analysis. However, on a minimal medium with or without L-phenylalanine, no gliovirin was produced. Successive extracts from millet (*i.e.* hexane, ether, methanol and water) when added to the minimal medium failed to elicit synthesis of gliovirin (with or without L-phenylalanine), while the millet residue after extraction continued to provide a suitable medium for gliovirin synthesis.

A purified hemicellulose fraction which was low in nitrogen (4.6%) was prepared from millet. *G. virens* grown on a minimal medium containing this hemicellulose and L-phenylalanine produced gliovirin in satisfactory yields (1,220 μg/ml). Significant quantities of gliovirin were not synthesized in the absence of L-phenylalanine (65 μg/ml) nor was any detected when D-phenylalanine or L-tyrosine was used as a substrate. Thus, the hemicellulose fraction appears to contain a factor which is essential for *G. virens* to produce gliovirin. The identity of gliovirin was established by comparison with the HPLC retention time and UV spectrum of an authentic sample of gliovirin and by comparison of the ^{1}H NMR spectrum (DMSO-d_6) with the published spectrum[1].

To confirm that L-phenylalanine was incorporated into gliovirin, L-[1-^{13}C]phenylalanine (20% 1-^{13}C) was used in the culture medium. The identity of gliovirin isolated from this experiment was confirmed as above. Because of the small amount of gliovirin isolated, only two peaks were observed in the ^{13}C NMR (acetone-d_6) at δ 164.6 and 167.0 corresponding to the two carbonyl groups in gliovirin. These observations confirm our previous hypothesis that both halves of the diketopiperazine ring in gliovirin originate from L-phenylalanine.

The ^{1}H NMR spectrum of gliovirin in DMSO allows one to study the incorporation into the right half of the gliovirin molecule (*i.e.* carbons 2, 3, and 12 to 18). The protons on C-2 and -12 appear at δ 4.47 and 4.42, respectively. Coupling between these protons is 1.2 Hz. In the ^{1}H NMR spectrum of gliovirin obtained from 20% 1-^{13}C-phenylalanine, the proton on C-2 appears as a doublet of doublets due to the coupling indicated above and to coupling to the C-3 carbonyl derived from the ^{13}C-labeled phenylalanine (J_{C-CH} = 7.2 Hz). These peaks are not fully resolved at 300 MHz, but deconvolution indicates 26% incorporation from 1-^{13}C-phenylalanine.

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