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OCCURRENCE AND BIOSYNTHESIS OF ASPERPHENAMATE IN SOLID CULTURES OF *PENICILLIUM BREVICOMPACTUM*

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Key Word Index—*Penicillium brevicompactum*; Hyphomycetes; fungus; biosynthesis; asperphenamate; phenylalanine derivatives.

Abstract—The ester of *N*-benzoylphenylalanine and *N*-benzoylphenylalaninol, asperphenamate, was isolated from solid cultures of *Penicillium brevicompactum*. Isotope from L-[U-^{14}C]phenylalanine was well incorporated into both benzoyl groups and into the phenylalanine and phenylalaninol moieties. Isotope from [U-^{14}C]benzoic acid was also well incorporated into asperphenamate.

INTRODUCTION

N-Benzoylphenylalanine and *N*-benzoylphenylalaninol are produced by shake cultures of *Penicillium brevicompactum* [1]. The latter is also a metabolite of *Aspergillus flavipes* [2] and of two higher plants [3, 4]. In *P. brevicompactum* radioactivity from L-[U-^{14}C]phenylalanine is efficiently incorporated into the C_6–C_1 and C_6–C_3 units of both benzamides [1]. The ester of *N*-benzoylphenylalanine and *N*-benzoylphenylalaninol, asperphenamate, is found in shake cultures of *A. flavipes* [2] and in surface cultures of *P. canadense* [5]. Traces of asperphenamate, insufficient for convenient biosynthetic work, are detectable in shake cultures of *P. brevicompactum* [1]. However, during studies of the correlation of secondary metabolism with the development cycle of *P. brevicompactum* in solid culture [6–8], appreciable quantities of asperphenamate (rather than of its component parts) were encountered. Using solid culture, the biosynthesis of asperphenamate has been examined.

RESULTS

The formal characterization of asperphenamate as a metabolic product of *P. brevicompactum* was first undertaken. *P. brevicompactum* was grown for 7 days on the surface of a dialysis membrane placed over Czapek–Dox agar [6]. This procedure allowed the biomass to be separated easily from the growth medium. Using the extraction methods of McCorkindale *et al.* [5], a material was isolated from the biomass that was identical in all respects to an authentic sample of asperphenamate. Asperphenamate represented *ca* 0.08% of the wet wt of a *P. brevicompactum* 'over' culture.

In solid cultures of *P. brevicompactum*, asperphenamate synthesis begins when an aerial mycelium is forming [8]. Culture conditions can be manipulated such that aerial hyphae develop vigorously and synchronously over the surface of a solid culture. This involves growing the fungus first between two sheets of dialysis membrane placed on top of nutrient agar ('between' culture); 48–144 hr subsequently, the upper sheet of membrane is stripped off leaving the biomass on the lower sheet of membrane which in turn remains on top of the agar (see ref. [6] for further details). Such 'stripped-between' cultures begin forming an aerial mycelium concertedly within 2 hr of stripping.

A set of *P. brevicompactum* 'between' cultures was stripped when they were 72 hr old. L-[U-^{14}C]Phenylalanine was administered to the cultures 9 hr after stripping. Asperphenamate was isolated 15 hr later by dilution analysis and was found to contain 5.9% of the added radioactivity. Base-catalysed hydrolysis of asperphenamate yielded *N*-benzoylphenylalanine and *N*-benzoylphenylalaninol. These benzamides contained 44.7 and 54.1% respectively of the total radioactivity of the parent ester. Removal of the benzoyl group of each benzamide established that this moiety represented 63.2% of the radioactivity of the *N*-benzoylphenylalanine and 54.2% of that of the *N*-benzoylphenylalaninol. The radioactivity incorporated into asperphenamate from [U-^{14}C]phenylalanine is therefore distributed between its component parts in the ratio: C_6–C_1 unit of the acid portion : C_6–C_3 unit of the acid portion : C_6–C_1 unit of the alcoholic portion : C_6–C_3 unit of the alcoholic portion (0.28 : 0.16 : 0.29 : 0.25).

When [U-^{14}C]benzoic acid was administered to solid cultures of *P. brevicompactum* that were active in asperphenamate synthesis, radioliquid chromatography established that 10.9% of the administered isotope was incorporated into asperphenamate.

DISCUSSION

When a solid culture of *P. brevicompactum* develops an aerial mycelium, four equivalents of phenylalanine are converted into the water-insoluble diamide ester, asperphenamate. In all likelihood the phenylalanine units derive from the significant proteolytic capacity that appears in *P. brevicompactum* cultures as aerial hyphae develop [Campbell, I. M. *et al.*, unpublished results]. The manner in which phenylalanine is converted to benzoic acid, and the sequence in which the components of asperphenamate are assembled, remain to be seen. Our original suggestion, based on study of the incorporation of radioactivity into *N*-benzoylphylalanine and *N*-benzoylphenylalaninol, that carbon for the construction of each benzamides was removed from the phenylalanine pool around the same time [1], is not supported by the current data.

EXPERIMENTAL

Isolation and characterization of asperphenamate. 1 l. Czapek–Dox agar growth medium (Difco Labs, Detroit, Michigan, U.S.A.) was placed in each of two aluminum turkey roasting pans (18 × 12 × 4 inches). The pans were covered with aluminium foil and sterilized by autoclaving. When cool, the surface of the agar was covered with overlapping strips of sterile dialysis membrane (18 × 3 inches, A. H. Thomas, Philadelphia, Pennsylvania). Spores of *P. brevicompactum* (ATCC 9056) were inoculated onto the surface of the dialysis membrane as an aq. suspension (*ca* 10^8 spores in 10 ml H_2O) and the 'over' culture [6] was allowed to grow at 23° for 7 days. The biomass (75.3 g) was isolated by peeling it and the dialysis membrane from the agar surface and then scraping the biomass from the membrane surface. The biomass was homogenized in $CHCl_3$ (1.5 l.), and the asperphenamate (60.9 mg) was isolated by chromatography (SiO_2, $CHCl_3$–EtOH, 20:1) and crystallization (EtOH), mp 206–207°, lit. [5] 210°; $CD[\theta]_{227} - 2.4 \times 10^4$, lit. [5] −22700. Spectral (mass and NMR) and chromatographic (TLC, HPLC) properties were identical to those of an authentic sample of asperphenamate provided by N. J. McCorkindale [5].

Biosynthesis of asperphenamate. Between cultures of *P. brevicompactum* [6] were established in Petri plates (10 cm) containing Czapek–Dox–glucose agar (20 ml) and were allowed to grow at 25° for 72 hr before being stripped. 9 hr following stripping, 3 plates were sprayed carefully [6] with L-[U-^{14}C]phenylalanine (450 μCi/μmol, 18.75 μCi/plate) and were allowed to incubate with the tracer for 15 hr. Thereafter, the biomass (1.4 g) was combined and was homogenized in $CHCl_3$–H_2O (pH 10; 1:1, v/v) in the presence of cold asperphenamate (68.8 mg). The asperphenamate (69.1 mg) was recovered from the organic phase, was purified chromatographically and was crystallized to a constant sp. act. of 1.06×10^5 dpm/mg. This corresponds to an *I*% value for the phenylalanine–asperphenamate conversion of 5.9%.

Asperphenamate (66.7 mg) was cleaved to *N*-benzoylphenylalanine and *N*-benzoylphenylalaninol by treatment with 0.07 M NaOH (18.6 ml) and EtOH (30 ml) at reflux for 28 hr. Direct extraction (EtOAc) of the reaction mixture yielded the alcohol; acidification of the residue and re-extraction yielded the acid. These were crystallized to constant sp. act. as described previously [1]; sp. act., *N*-benzoylphenylalanine: 2.40×10^4 dpm/μmol, *N*-benzoylphenylalaninol: 2.90×10^4 dpm/μmol.

Following dilution of each, the benzoyl group was removed from each benzamide as described before [1]. *N*-Benzoylphenylalanine (starting sp. act. 2607 dpm/μmol) gave rise to benzoic acid of sp. act. 1647 dpm/μmol; *N*-benzoylphenylalaninol (starting sp. act. 2476 dpm/μmol) gave rise to benzoic acid of sp. act. 1342 dpm/μmol.

A second biosynthetic expt was run with [7-^{14}C]benzoic acid (22.6 μCi/μmol, 5 μCi to each of three stripped-between cultures). The protocol was as described above, except that unlabelled carrier asperphamate was not added; rather the mass and radioactivity of asperphenamate were assayed by radioliquid chromatography (Berthold LB 503/Perkin Elmer series 2 system) on a C-18 reversed phase column in the solvent system 20% H_2O in MeOH (radio counter efficiency 18%). The recovered asperphenamate had a sp. act. of 2.2×10^6 dpm/μmol; [7-^{14}C]benzoic acid was incorporated into asperphenamate with an efficiency of 10.9%.

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