Aspergillic Acids Produced by Mixed Cultures of *Aspergillus flavus* and *Aspergillus nidulans*

By M. J. PERRY,† J. F. MAKINS,‡ M. W. ADLARD* AND G. HOLT

*The Polytechnic of Central London, School of Engineering and Science,
115 New Cavendish Street, London W1M 8JS, UK*

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A mixed culture of *Aspergillus nidulans* (GH79) and *Aspergillus flavus* (CMI 91019B) produced two antibiotics, designated VI and VII, which were not elaborated when either fungus was grown alone. Chemical and spectroscopic analysis of VI, the major component, indicated that this compound was identical to hydroxyaspergillic acid. The minor component, VII, was produced in too low a yield for its identity to be established. However, partial characterization suggests that this antibiotic also belongs to the aspergillic acid group of mycotoxins.

INTRODUCTION

Our laboratory has for some time been engaged in a study of antibiotic biosynthesis by individual strains of *Aspergillus nidulans* (Cole *et al.*, 1976; Holt *et al.*, 1976; Makins *et al.*, 1980; Perry *et al.*, 1982*a, b*). However, there is scant information in the literature concerning the use of mixed cultures of aspergilli to produce new metabolites. This is probably due to the difficulty in obtaining stable, mixed microbial systems suitable for subsequent commercial development. However, the progress made in recombinational techniques (Ferenczy, 1979; Hopwood & Chater, 1980; Holt & Saunders, 1983, 1984) means that it is now feasible to obtain recombinants of genetically diverse fungi. From such hybrids strain improvement procedures could lead to the production of the new antibiotics at a commercially acceptable level. If hybridization experiments are to be carried out in a search for new metabolites then an initial problem is to decide which strains should be recombined (Holt & Saunders, 1983). This paper describes one approach, whereby mixed cultures are screened for activity not shown by either of the component strains. Isolates of *A. nidulans* and *A. flavus* were chosen as a model system since strains of each genetically labelled on eight linkage groups are available for possible detailed study of interspecific recombination.

METHODS

Strains and culture conditions. The genotype and origins of the strains of *Aspergillus nidulans* (GH79) and *A. flavus* (CMI 91019B) employed are given in Table 1. *Aspergillus nidulans* carried a number of genetical markers including the mutation *npeA* which results in impaired penicillin production (Holt *et al.*, 1976). A prototrophic strain of *A. flavus* was used.

Culture conditions, media and levels of nutritional supplements for routine growth were based on those of Pontecorvo *et al.* (1953) as adapted by Clutterbuck (1974). Submerged liquid cultures for antibiotic production were shaken at 25 °C on an orbital shaker (200 r.p.m., 5 cm throw) in 100 ml polypropylene flasks containing 20 ml of the antibiotic fermentation medium described by Holt & Macdonald (1968) but lacking the penicillin side chain precursor, phenylacetic acid. Except where stated, inoculations were made with 1 ml of spore

† Present address: National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3, UK.

‡ Present address: Genetic Engineering Research Department, Bristol Myers Co., PO Box 657, Syracuse, New York 13201, USA.

Table 1. *Details of micro-organisms used*

Organism	Origin*	Genotype
<i>Aspergillus flavus</i>	CMI 91019B	Prototrophic
<i>Aspergillus nidulans</i>	PCL (GH79)	<i>yA pyroA cnxA npeA</i> †
<i>Bacillus subtilis</i>	NCTC 8236	Prototrophic strains of test organisms used for the antibiotic bioassay
<i>Escherichia coli</i>	NCTC 86	
<i>Proteus vulgaris</i>	NCTC 4635	
<i>Pseudomonas aeruginosa</i>	NCIB 8295	
<i>Serratia marcescens</i>	NCTC 8706	
<i>Staphylococcus aureus</i>	ATCC 27735	

* CMI, Commonwealth Mycological Institute; PCL, Polytechnic of Central London; NCTC, National Collection of Type Cultures, UK; NCIB, National Collection of Industrial Bacteria; ATCC, American Type Culture Collection.

† Gene symbols: *yA*, yellow conidia; *pyroA*, auxotrophic requirement for pyridoxin; *cnxA*, requirement for reduced nitrogen; *npeA*, impaired in penicillin production.

suspensions containing approximately 10^7 spores ml^{-1} . Bioassays of culture filtrates were performed according to the procedures outlined by Brownlee *et al.* (1948) and included β -lactamase (Sigma) in the agar at a final concentration of 80 units ml^{-1} in order to hydrolyse any β -lactam antibiotics produced.

Purification procedure. Compounds VI and VII were extracted from the fermentation liquor (pH 7.0) into ethyl acetate. The solvent extract was concentrated *in vacuo* and applied to a column of silica gel. The column was developed with ethyl acetate to elute compound VI, then the solvent was changed to ethyl acetate/methanol (75:25, v/v) to elute compound VII.

Those fractions containing compound VI were pooled and the solvent removed *in vacuo*. The preparation was dissolved in 0.1 M- Na_2CO_3 , extracted with benzene and the solvent layers discarded. The aqueous phase was acidified with HCl and extracted with ethyl acetate. Removal of the solvent *in vacuo* yielded compound VI as a yellow crystalline product which was homogeneous under TLC. A crude melting point of 142–145 °C was recorded for the product, which was obtained in too low a yield to permit recrystallization.

After fractionation over silica gel, the fractions containing compound VII were combined and the solvent removed *in vacuo*. The preparation was then dissolved in methanol and applied to a column of Sephadex LH-20 developed with the same solvent. Those fractions containing compound VII were pooled and removal of the solvent *in vacuo* yielded this antibiotic as a yellow crystalline product, crude melting point 93–98 °C, which was homogeneous on TLC but could not be recrystallized due to the low yield.

Gas-liquid chromatography. This was carried out on a Perkin Elmer F11 chromatograph equipped with an FID detector and a 1 m OV1 column. The oven temperature was initially 200 °C and was programmed to increase at 5 °C min^{-1} . Injection temperature was approximately 280 °C, the carrier gas was nitrogen at 20 ml min^{-1} , the range was 10, and the attenuation was 16 with an injected sample of 2 μl .

Trimethylsilyl derivatives of compound VI and neohydroxyaspergillic acid were prepared by reacting 1.5 mg of each with 0.5 ml Regisil RC-2 (Phase Separations Ltd, Deeside Industrial Estate, Queensferry, Clwyd CH5 2LR, UK) at 120 °C for 2 h.

RESULTS

A strain of *A. nidulans* (GH79) impaired in penicillin production was chosen as one of the component cultures, in order to facilitate recognition of antibiotic activity other than β -lactams even when tested against β -lactam sensitive bacteria. It was grown in mixed culture with a prototrophic strain of *A. flavus* (CMI 91019B). This combination yielded an activity against *Escherichia coli* and *Staphylococcus aureus* which was not present when either fungus was grown alone. Peak titres were observed after 6 d growth. Examination of the culture filtrate revealed the presence of two antibiotics designated VI and VII (V and other antibiotics produced by strains of *A. nidulans* have been described elsewhere: Cole, 1980; Cole *et al.*, 1976; Middleton *et al.*, 1979; Perry *et al.*, 1982a, b).

Chemical properties. Compounds VI and VII both formed red-coloured complexes with ferric chloride and green-coloured cupric salts, which is consistent with their possessing hydroxamic acid structures. They were also resistant to acidic and alkaline hydrolysis, suggesting that the hydroxamic acid group is part of a heterocyclic system and not of the simple aliphatic type.

Fig. 1. Mass spectrum of compound VI.

Antibiotic activity. No minimum inhibitory concentration (MIC) values were obtained for compound VII, although this antibiotic has been observed to inhibit the growth of *Bacillus subtilis*, *E. coli* and *S. aureus*. MIC values for compound VI were obtained using an agar plate diffusion assay in which 1 cm diameter discs of filter paper (Whatman no. 1) were impregnated with compound VI. The agar used was diagnostic sensitivity test agar (Oxoid) which had an iron content of $1.2 \mu\text{g ml}^{-1}$. The MIC values were ($\mu\text{g ml}^{-1}$): *B. subtilis*, 26; *E. coli*, 14; *Pseudomonas aeruginosa*, 70; *Proteus vulgaris*, 98; *Staphylococcus aureus*, 30; *Serratia marcescens*, > 100.

Mass spectroscopy. Mass spectrometry of compound VI was done by the Physico-Chemical Measurements Unit, Harwell, Berks., UK. The molecular ion was at m/e 240.1481 and the suggested formula was $\text{C}_{12}\text{H}_{20}\text{O}_3\text{N}_2$ (see Fig. 1).

Infrared spectroscopy. Compound VI in a KBr disc exhibited the following absorption maxima: 2945, 2920, 2880, 1580, 1530, 1410, 1230, 1160, 1120, 1040 and 1000 cm^{-1} .

Ultraviolet spectroscopy. In ethanol, the UV spectra of compounds VI and VII were very similar, exhibiting absorptions centred at 212, 232 and 333 nm.

The chemical and spectroscopic analysis of compound VI strongly indicated that it was a hydroxylated member of the aspergillic acid group of mycotoxins known to be produced by several *Aspergillus* species (Steyn, 1980). Compound VI was therefore compared with an authentic sample of neohydroxyaspergillic acid kindly supplied by Dr L. Delcambe, International Centre of Information on Antibiotics, Boulevard de la Constitution 32, B-4020 Liège, Belgium. Although the two components were indistinguishable by TLC, they were well resolved by GLC. The tetramethylsilyl derivative of component VI had a retention time of 6.4 min compared with 5.5 min for TMS-neohydroxyaspergillic acid. The mass spectrum of component VI differed significantly from that reported for β -hydroxyneaspergillic acid (Mabayashi *et al.*, 1978). According to Steyn (1980), therefore, three structures were likely (Fig. 2). The mass spectrum of compound VI showed prominent peaks at m/e 195, 182, 153, 43, 29 and 27 which may arise thus: 195, loss of O from M^+ (240) followed by loss of C_2H_5 ; 182, loss of O from M^+ followed by loss of C_3H_6 by McLafferty rearrangement; 153, loss of C_2H_5 from 182 or C_3H_6 from 195 by McLafferty rearrangement; 43, C_3H_7^+ ion; 29, C_2H_5^+ ion; 27, C_2H_3^+ ion.

Structure (I) was discounted as the loss of C_2H_5 from m/e 182 to give m/e 153 was not possible. As (III) was thought unlikely to give rise to C_3H_7 , compound VI was probably hydroxyaspergillic acid (II). In addition, the crude melting point obtained for compound VI ($142\text{--}145^\circ\text{C}$) was consistent with that reported for hydroxyaspergillic acid ($148\text{--}150^\circ\text{C}$) (Steyn, 1980).

Fig. 2. Structural formulae of hydroxyaspergillic acids. The compounds are shown in the hydroxamic acid tautomeric forms which are believed to be responsible for their antibiotic properties.

The production of aspergillic acids by fungi in liquid culture is known to be both strain- and medium-specific (Middleton *et al.*, 1979) and to be influenced by the size of the inoculum. Thus the detection of antibiotic activity could have resulted from an increase in the innate production of some hydroxamic acid by one strain from a level below, to one above, the sensitivity of the bioassay system as a result of the physiological conditions of the culture rather than a *de novo* biosynthetic reaction between the two species. To test this possibility two further experiments were performed. (a) Pure cultures of *A. nidulans* GH79 and *A. flavus* CMI 91019B were tested for activity against *E. coli* after 6 d submerged shaken culture in 20 ml fermentation medium inoculated with different amounts of fungal conidia (from 10^2 to 10^9). (b) Filtrates (100 ml) of pure cultures were extracted with 100 ml chloroform, and the extract was evaporated to dryness *in vacuo*, redissolved in 10 ml water and tested in bioassay against *E. coli*. This procedure would have concentrated tenfold any aspergillic acids present. Under each condition neither fungal strain alone produced an antibiotic active against *E. coli*.

DISCUSSION

A mixed culture of *A. nidulans* GH79 and *A. flavus* CMI 91019B produced two antibiotics, VI and VII, which were not elaborated by pure cultures of either strain. Chemical and spectroscopic analysis of compound VI strongly suggested that this antibiotic was hydroxyaspergillic acid. Unfortunately compound VII was not produced in sufficient quantity for a detailed analysis to be feasible. However, it gave a typical hydroxamate reaction with both ferric and cupric chloride and had a UV spectrum similar to that of compound VI. We therefore believe that compound VII is also probably a member of the aspergillic acid group of metabolites. Anke (1977) has shown that the activity of hydroxamic acid antibiotics is sensitive to environmental iron levels. However, compounds VI and VII were both produced in a complex fermentation medium with an unknown iron content.

Although aspergillic acid and its derivatives are known to be produced by certain strains of *A. flavus* (Steyn, 1980) they have not been isolated from *A. nidulans*. The mechanisms by which the biosynthetic pathways of *A. nidulans* GH79 and *A. flavus* CMI 91019B interact to produce compounds VI and VII are unknown but could include: (1) the biochemical transformation of metabolites of one species by the other; (2) deregulation or suppression of biosynthetic pathways; and (3) complex cosynthetic interactions.

The results presented here demonstrate the feasibility of obtaining detectable levels of interesting metabolites from mixed cultures, which could thus provide a new source of biologically useful molecules. Studies on the generation of such metabolic novelty, by hybridization using protoplast fusion of genetically marked strains of *Aspergillus* species, are underway.

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