# A HYDROXAMIC ACID FROM *ASPERGILLUS NIDULANS* WITH ANTIBIOTIC ACTIVITY AGAINST *PROTEUS* SPECIES

A. J. MIDDLETON*, D. S. COLE** and K. D. MACDONALD***

Microbiological Research Establishment, Porton Down, Salisbury
Wiltshire, SP4 OJG, England

(Received for publication July 17, 1978)

An iron-complexing antibiotic with a narrow spectrum of biological activity was produced by several strains of *Aspergillus nidulans* when grown in a low-iron, chemically defined medium. Its chemical and biological properties closely resembled those of desferritriacetylfusigen, a metabolite of several other *Aspergilli* and *Penicillia*.

The ascomycete fungus *Aspergillus nidulans* is known to elaborate a number of secondary metabolites, some of which possess antibiotic activity. The latter include benzylpenicillin[1], cordycepin[2], asperline[3] and echinocandin B[4]. The ability to produce antibiotics is both strain-specific and dependent upon fermentation conditions[5]. As part of a genetic investigation of antibiotic production in *A. nidulans*, a large number of strains were tested and some had antibiotic activity not attributable to any of the above products[6]. Detailed studies were made of an antibiotic, designated V, from one of these strains.

## Materials and Methods

Unless otherwise indicated, chemicals and solvents were of analytical grade and supplied by BDH Chemicals Ltd., Poole, England.

### Media

Strains of *A. nidulans* were grown as slant cultures on the complete medium (CM) agar given by MACDONALD, HUTCHINSON and GILLETT[7]. The medium used for production of antibiotic V was based on that of ANKE[8] and had the following composition: glucose, 50 g; L-asparagine, 10 g; $KH_2PO_4$, 5 g; $MgSO_4 \cdot 7H_2O$, 5 g; liquid CM, 2 ml; deionised water to 1 liter; pH 7.0. This 'low-iron' medium contained 0.15~0.2 $\mu$g/ml of iron.

Two types of media were used for microbial growth when determining the antibiotic spectrum of V: a complex medium, Diagnostic Sensitivity Test agar (DST), which was Oxoid medium No CM261; its iron content was 1.2 $\mu$g/ml: a minimal medium (MM) made up as follows: Solution A: Oxoid agar No L28, 6 g; trisodium citrate, 0.2 g; deionised water to 200 ml; autoclaved 115°C for 15 minutes. Solution B: $K_2HPO_4$, 21 g; $KH_2PO_4$, 9 g; trisodium citrate, 0.94 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; deionised water to 1 liter; pH 7.2; autoclaved 115°C for 15 minutes. Solution C: 50% (w/v) glucose solution in deionised water; autoclaved 115°C for 10 minutes. The final medium contained Solution A, 200 ml; Solution B, 200 ml; Solution C, 16 ml. It had an iron content of 0.7 $\mu$g/ml.

### Measurement of Absorption Spectra

Infrared spectra were determined in KBr discs using a Perkin-Elmer 157 infrared spectrometer. Proton magnetic resonance spectra were measured in deuterated chloroform on a Perkin-Elmer 60 MHz spectrometer; tetramethylsilane was used as an internal standard. A Unicam SP 1800 instrument

* Present address: G. E. C. Marconi (Radar Division), New Parks, Leicester, England.
** Present address: Smith Kline and French Laboratories, Welwyn Garden City, Hertfordshire, England.
*** To whom correspondence should be addressed.

was employed for ultraviolet absorption measurements.

Column Chromatography

Sephadex LH20 for gel permeation chromatography was obtained from Pharmacia Fine Chemicals, London, England. Silica gel for adsorption chromatography (Kieselgel 60 Art 7754) was supplied by Merck, Darmstadt, West Germany. It was activated by heating at 110°C for 24 hours and then partially deactivated by the addition of 6% (w/w) deionised water.

Thin-Layer Chromatography (TLC)

Aluminium backed TLC sheets, obtained from Merck, Darmstadt, West Germany, were used without prior activation. Five solvent systems were employed as shown in Table 1.

Table 1. Comparison of iron (III)/V with triacetylfusigen (TAF) in silica gel thin-layer chromatography

| Solvent systems, composition (v/v) | Rf×100 | |
|---|---|---|
| | Iron (III)/V | TAF |
| Chloroform - methanol - water (65: 25: 4) | 56 | 56 |
| *n*-Propanol - glacial acetic acid - water (4: 1: 1) | 40 | 40 |
| *n*-Butanol - glacial acetic acid - water (4: 1: 1) | 23 | 23 |
| Chloroform - *n*-propanol - methanol - water (18: 7: 2: 1) | 28 | 28 |
| Chloroform - benzyl alcohol - methanol (2: 1: 1) | 57 | 57 |

Addition and Removal of Iron

The Iron (III)/V complex was formed by a modification of the method of MOORE and EMERY[9]. Excess $FeSO_4 \cdot 7H_2O$ was added to an aqueous solution of V and the resulting mixture aerated for three hours at room temperature by agitation on a reciprocal shaker. Ferric ion produced by the aerial oxidation of ferrous ion combined with V to produce the red-brown complex which was purified by extracting into chloroform. Removal of ferric ion from triacetylfusigen was achieved by the method of DIEKMANN and ZAHNER[10].

## Results

### Production and Isolation of Antibiotic V

The strain of *A. nidulans* used was a green-sporing prototroph, designated MRE 628. Suspensions of conidia from CM agar slants were inoculated into 100-ml conical polypropylene shake flasks containing 20 ml of the "low-iron" medium. Flasks were incubated at 25°C on a rotary shaker running at 220 rpm with a 5-cm throw.

Antibiotic concentration, monitored by agar diffusion assay against *Proteus vulgaris* strain MRE 1127, reached a maximum of about 100 mg/liter after 4 days. Shake flask cultures were harvested by filtration through muslin and the mycelium discarded. The antibiotic V was extracted into chloroform at pH 4 and after drying over anhydrous sodium sulphate, the solvent removed under vacuum. The concentrate was dissolved in methanol, applied to a Sephadex LH20 column and eluted with methanol. Active fractions were detected by the red-brown coloration produced on adding $FeCl_3$. Final purification was achieved by adsorption onto silica gel in a solvent of 15% (v/v) methanol in ethyl acetate, and eluting with the same solvent.

In a typical extraction, 450 mg of V were obtained from 10 liters of culture filtrate (yield 45%). This preparation when converted to the iron (III) complex was homogeneous on silica gel TLC in five solvent systems (see later section).

Subsequently, 83 natural isolates of *A. nidulans* were grown under the above conditions. Culture filtrates of 63 gave antibiotic activity against *P. vulgaris*. Five of these, University of Birmingham isolates 31, 33, 43, 51 and 138, were examined further; all synthesised a compound chromatographically indistinguishable from the antibiotic elaborated by strain MRE 628. The ability to synthesise V is clearly widespread among natural isolates of *A. nidulans*.

Fig. 1. Infrared spectrum of Fe(III)/V complex determined in KBr disc.

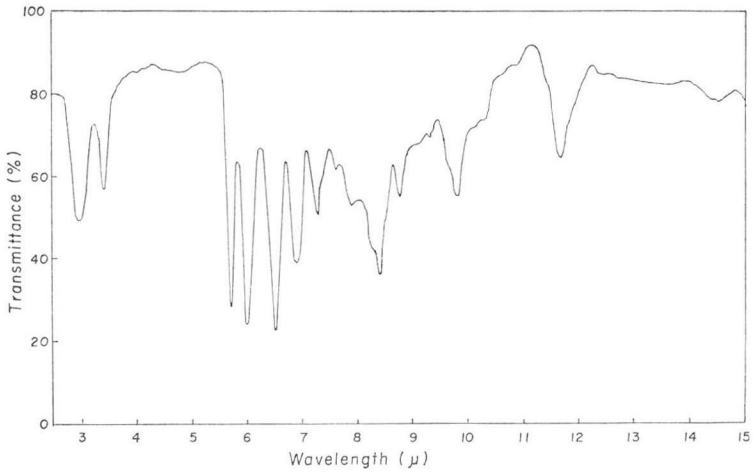
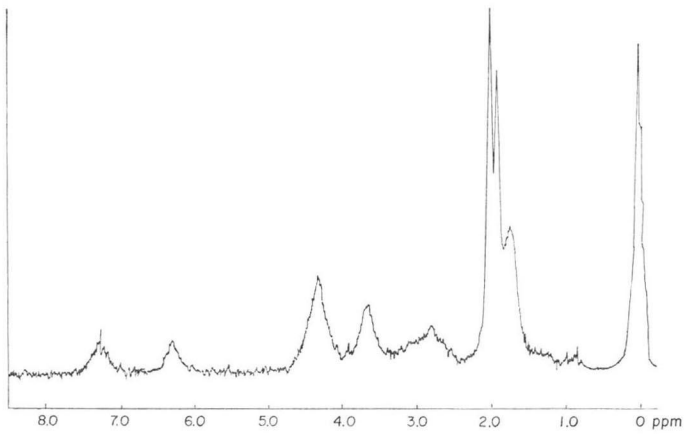



Fig. 2. Proton magnetic resonance spectrum of antibiotic V determined in deuterated chloroform.




## Physical and Chemical Properties of Antibiotic V

The antibiotic designated V is a white, non-crystalline glass-like solid. It was soluble in a variety of solvents, including water, methanol and chloroform. The addition of ferric ions produced a red-brown complex with a broad absorption peak at about 440 nm. This complexing behaviour is characteristic of a group of natural products known as hydroxamic acids, found widely as products of fungi and actinomycetes[11]. The absorption maximum of the iron (III)/V complex was very resistant to pH changes, remaining unaltered at pH 2. This stability is characteristic of a hydroxamic acid in which 3 hydroxamate groups occur in the same molecule, leading to the formation of a very stable hexadentate ferric complex. Many siderochromes are hydrophilic compounds soluble only in very polar solvents. The unusual lipophilic character of V, evidenced by its solubility in chloroform and the ease with which the iron (III)/V complex could be extracted from aqueous solution into *n*-butanol or chloroform resembles the behaviour of desferritriacetylfusigen (DFTAF) and its corresponding iron (III) complex, triacetylfusigen (TAF). The following comparisons were made.

Infrared Spectrum: The IR spectrum of the iron (III)/V complex shown in Fig. 1 is identical to

Table 2. Antimicrobial activity of V compared with desferritriacetylfusigen (DFTAF)

| Organism | Minimum inhibitory level ($\mu$g) | | | |
|---|---|---|---|---|
| | DST agar* | | MM agar* | |
| | V | DFTAF | V | DFTAF |
| *Bacillus brevis* ATCC 9999 | <10 | 10~20 | (no growth) | |
| *Bacillus subtilis* NCTC 8236 | >150 | >50 | 5~10 | 10~20 |
| *Staphylococcus aureus* ATCC 27735 | 5~10** | 10~20** | (no growth) | |
| *Proteus vulgaris* NCTC 4635 | 5~10 | <10 | (no growth) | |
| *Proteus vulgaris* MRE 1127 | 5~10 | <10 | (no growth) | |
| *Proteus mirabilis* MRE 402 | 5~10 | <10 | (no growth) | |
| *Proteus morganii* MRE 630 | 5~10 | — | (no growth) | |
| *Escherichia coli* MRE 163 | >150 | >50 | >150 | >50 |
| *Serratia marcescens* MRE 619 | >150 | >50 | >150 | >50 |
| *Klebsiella aerogenes* MRE 0013 | >150 | — | >150 | >50 |
| *Salmonella typhimurium* MRE 00310 | >150 | — | >150 | >50 |
| *Pseudomonas aeruginosa* NCTC 6750 | >150 | — | — | — |
| *Sarcina lutea* NCIB 8553 | >150 | >50 | (no growth) | |
| *Candida albicans* MRE 00214 | >150 | — | >150 | — |

Assays were performed in bioassay plates (Nunc, Denmark) containing 100 ml of agar seeded with overnight broth cultures of organisms grown in nutrient broth (for DST plates) or mineral salts+glucose (for MM plates). Whatman paper discs, 8 mm in diameter, were impregnated with 1~150 $\mu$g of V or 10~50 $\mu$g of DFTAF and placed on the surface of the agar in bioassay plates. After storage for 2 hours at 4°C, plates were incubated overnight at 30°C for *B. brevis* and at 37°C for all other organisms.

* Iron contents: DST; 1.2 $\mu$g/ml, MM; 0.7 $\mu$g/ml.

** Partial inhibition only.

the published spectrum of TAF[12].

Proton Magnetic Resonance Spectrum: The spectrum of V dissolved in deuterated chloroform and illustrated in Fig. 2 is similar to the published spectrum of DFTAF determined under identical conditions[10].

Silica Gel Thin-Layer Chromatography: The iron (III)/V complex chromatographed as a single spot in 5 solvent systems. A comparison with TAF under the same conditions showed that the Rf values were identical for both compounds (Table 1). Furthermore a mixture of iron (III)/V and TAF was indistinguishable in all 5 solvent systems from TAF alone.

Sephadex LH20 Chromatography: The elution pattern of a mixture of TAF and iron (III)/V was identical to that of either compound alone.

Elemental Analysis: V (found): C, 54.63%; H, 7.13%; N, 9.70%. DFTAF (calculated for $C_{39}H_{60}N_6O_{15}$): C, 54.93%; H, 7.04%; N, 9.86%.

## Biological Properties of Antibiotic V

The antibiotic activities of V and DFTAF were compared against microorganisms grown in the complex medium (DST) and chemically defined minimal medium (MM) given above (Table 2). In DST agar, both were active against *Bacillus brevis* and all the *Proteus* species tested, and *Staphylococcus aureus* was also partially inhibited by low levels of each hydroxamate. Although *Bacillus subtilis* was insensitive in DST, it became sensitive to both antibiotics after growth in MM. Neither TAF nor the iron (III)/V complex displayed antibiotic activity against any of the organisms tested.

The antimicrobial spectrum of DFTAF has been reported by ANKE[8] who tested this compound in a complex and a minimal medium; the latter was defined as "iron-free". Activity was limited to a small number of Gram-positive and Gram-negative bacteria in complex medium, but extended to a

wider range of bacterial species in minimal medium. Comparison of our results with those of ANKE[8] is difficult because iron levels in the complex and defined media used by ANKE were not given. Also, in every case except *B. brevis* different strains of the test species were employed. However, it is clear that V and DFTAF have very similar antimicrobial spectra when tested under identical conditions (Table 2).

## Discussion

The antibiotic V from *A. nidulans* is similar, and probably identical, to desferritriacetylfusigen. The latter was first reported as a metabolite of 4 *Aspergilli* from the *Aspergillus fumigatus* taxonomic group: *A. fumigatus* mut. *helvolus*, *A. fumigatus* var. *ellipticus*, *A. breviceps* and *A. aureolus*[12]. MOORE and EMERY[9] described the production of DFTAF and other $N^{\alpha}$-acetylfusarinines by a species of *Penicillium* grown in a low-iron medium. This metabolite has subsequently been identified by ANKE[8] among the antibiotics elaborated by *Aspergillus deflectus*, a member of the *A. ustus* taxonomic group. ANKE[8] also reported DFTAF to be produced by other, unspecified, *Penicillia* and some Basidiomycetes. It is therefore, a metabolite of fairly widespread occurrence among fungi, especially *Aspergilli*, and its identification as a product of a member of the *A. nidulans* group is not surprising.

A major feature of the antimicrobial activity of DFTAF under the assay conditions described was its strong *in vitro* activity against *Proteus* species. The only other fungal trihydroxamic acid reported to have antibiotic activity is desferrioxamine B, which also inhibits *P. vulgaris*[13]. The mechanism of antibiotic action of DFTAF may involve the complexing of free iron. Other trihydroxamates could behave similarly since iron-binding is a feature of these compounds. The compound DFTAF may be considered a primary metabolite having a role in iron transport and possessing fortuitous antibiotic activity, rather than an atypical secondary metabolite.

Acknowledgements

This work was supported by the National Research Development Corporation in collaboration with the Procurement Executive, Ministry of Defence. We would like to thank Mrs. E. M. ROSS and Mr. C. GRANT for technical assistance, Dr. J. CHITTENDEN for NMR and IR spectra, Mrs. C. BARCLAY for elemental analysis and Mr. H. K. ROBINSON for iron estimations. We are very grateful to Prof. H. DIEKMANN and Dr. H. ANKE of the University of Tubingen, W. Germany, for the gift of authentic samples of triacetylfusigen, and copies of its infrared spectrum and to Prof. J. L. JINKS FRS of the University of Birmingham, England for providing strains of *Aspergillus nidulans*.

## References

1) HOLT, G. & K. D. MACDONALD: Penicillin production and its mode of inheritance in *Aspergillus nidulans*. Antonie van Leeuwenhoek 34: 409~416, 1968
2) KACKZA, E. A.; E. L. DULANEY, C. O. GITTERMAN, H. BOYD WOODRUFF & K. FOLKERS: Isolation and inhibitory effects on KB cell cultures of 3′-deoxyadenosine from *Aspergillus nidulans* (EIDAM) WINT. Biochem. Biophys. Res. Commun. 14: 452~455, 1964
3) ARGOUDELIS, A. D.; J. H. COATS & R. R. HERR: Isolation and characterisation of a new antibiotic, U-13,933. Antimicr. Agents & Chemoth. 5: 801~803, 1965
4) BENZ, F.; F. KNUSEL, J. NUESCH, H. TREICHLER, W. VOSER, R. NYFELLER & W. KELLER-SCHIERLEIN: Echinocandin B, a new polypeptide antibiotic from *Aspergillus nidulans* var. *echinulatus*. Helv. Chim. Acta 57: 2459~2477, 1974
5) COLE, D. S.; G. F. ST. L. EDWARDS, G. HOLT & K. D. MACDONALD: Antibiotic production by wild-type and mutant strains of *Aspergillus nidulans*. Abstracts, Fifth International Fermentation Symposium, p. 193. *ed* H. DELLWEG, 1976
6) COLE, D. S.: Antibiotic production in *Aspergillus nidulans*. Ph. D. Thesis, Polytechnic of Central London, 1977

7) MACDONALD, K. D.; J. M. HUTCHINSON & W. A. GILLETT: Isolation of auxotrophs of *Penicillium chrysogenum* and their penicillin yields. J. Gen. Microbiol. 33: 365~374, 1963
8) ANKE, H.: Metabolic products of microorganisms. 163. Desferritriacetylfusigen, an antibiotic from *Aspergillus deflectus*. J. Antibiotics 30: 125~128, 1977
9) MOORE, R. E. & T. EMERY: $N^{\alpha}$-Acetylfusarinines; Isolation, characterisation and properties. Biochemistry 15: 2719~2723, 1976
10) DIEKMANN, H. & H. ZAHNER: Konstitution von Fusigen und dessen Abbau zu $\Delta^{2}$-Anhydromevalonsaurelacton. Eur. J. Biochem. 3: 213~218, 1967
11) DIEKMANN, H.: Siderochromes iron (III)-trihydroxamates. *In* Handbook of microbiology. Vol. III. *ed.* A. I. LASKIN and H. A. LECHEVALIER, pp. 449~457, Chemical Rubber Company Press, Cleveland, Ohio, 1973
12) DIEKMANN, H. & E. KREZDORN: Metabolic products of microorganisms. 150. Ferricrocin, triacetylfusigen and other sideramines from fungi of the genus *Aspergillus*, group *fumigatus*. Arch. Microbiol. 106: 191~194, 1975
13) MARUYAMA, H. B.; H. AZUMA, Y. KOTOH & Y. SUHARA: Desferrioxamine B, an inhibitor of *E. coli* motility reversing the stimulating effect of cyclic adenosine-3′, 5′, monophosphate. Antimicr. Agents & Chemoth. 7: 377~388, 1975