UK-69,753, A NOVEL MEMBER OF THE EFROTOMYCIN FAMILY OF ANTIBIOTICS

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION AND ISOLATION

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UK-69,753 is a novel antibiotic structurally related to efrotomycin and factumycin, produced by a new strain of *Amycolatopsis orientalis* (ATCC 53550). The antibiotic potency in the fermentation broth was monitored by HPLC with diode array detection. A six tube counter current distribution was used to purify UK-69,753.

In the course of our screening programme for new antibiotics, *Amycolatopsis orientalis*, strain N731-15, was found to produce a complex of novel antibiotics structurally related to efrotomycin[1] and factumycin.[2] This paper describes the taxonomy of the producing organism and the fermentation and isolation of the major component UK-69,753.

Taxonomy of the Producing Strain N731-15

Bacterial Strain

The UK-69,753 producing strain, N731-15 was isolated from a soil sample collected from Yorkshire, England.

Morphological Characterisation

The morphological properties were observed on inorganic salts - starch agar, tap water agar and oatmeal agar after 14 days incubation at 28°C.

Cultural and Physiological Characterisation

The fifteen different media given in Table 1 were used for cultural and physiological characterisation. Colours were determined by comparison with colour chips from the Colour Harmony Manual.[3] Utilisation

of the carbon sources and biochemical tests was assessed by the methods of GORDON *et al.*[4], and SHIRLING and GOTTLIEB.[5]

Cell Wall and Cell Membrane Analysis

The methods used for whole-cell amino acid and sugar analyses are those described by BECKER *et al.*[6], and LECHEVALIER[7] respectively. Analysis of the wet mycelium for mycolates used the method of LECHEVALIER *et al.*[8] Phospholipid analysis of the cell membrane was determined as described by MINNIKIN *et al.*[9]

Results

Morphological Characteristics

Strain N731-15 failed to produce aerial mycelium or spores on any of the media used, even after incubation for 4 weeks. On inorganic salts - starch agar short chains of spore-like structures were produced along some segments of the substrate hyphae, but these might represent condensation of the cytoplasm. On tap water agar swellings developed at the tips of the substrate hyphae, which resembled spores. The vegetative hyphae on oatmeal agar were narrow, branched and measured 0.4 to 0.9 μm in diameter.

Cultural and Physiological Characteristics

Cultural characteristics of strain N731-15 grown in various media at 28°C for 14 days are shown in Table 1. Physiological characteristics of the strain are summarised in Table 2 and utilisation of carbon sources is shown in Table 3.

Strain N731-15 is characterised by cream to pale yellowish substrate mycelium, and the lack of aerial mycelium and spores. The whole-cell hydrolysates contain *meso*-diaminopimelic acid, galactose and arabinose, and are of type IV as defined by LECHEVALIER and LECHEVALIER.[10] Mycolates are absent but phospholipids, phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol are present which describes a type PII pattern. These characteristics together with the biochemical and physiological data in Tables 2 and 3 agree with the description of the genus *Amycolatopsis* recently proposed by LECHEVALIER *et al.*[11] However, there are notable exceptions from the type strain *A. orientalis* in decomposition of adenine, lack of esculinase, and no acid production from inositol, lactose and α-methyl-D-glucoside. Strain N731-15 is therefore considered as a new strain of *A. orientalis* (Pittenger and Brigham) LECHEVALIER *et al.*[11] It has been deposited at the American Type Culture Collection, Rockville, Maryland 20852, U.S.A. and assigned the accession No. ATCC 53550.

Fermentation

The inoculum was prepared by washing with 10 ml of sterile water, a slant culture of *A. orientalis*, ATCC 53550, maintained on a medium of glucose 4 g, yeast extract 4 g, malt extract 10 g, coconut milk 50 ml and agar 10 g in 1 litre of tap water, adjusted to pH 7.3 before autoclaving. The inoculum was transferred to an Erlenmeyer flask containing 100 ml of a medium of glucose 0.1 g, corn starch 2.4 g, peptone 0.5 g, yeast extract 0.5 g, beef extract 0.3 g, adjusted to pH 6.8, and incubated at 28°C on a rotary shaker with a 2.5-cm throw at 170 rpm for 48 hours. The resultant vegetative growth (50 ml) was used to inoculate two 3-litre Fernbach conical flasks each containing 1 litre of the above seed medium for preparation of the second seed culture. These flasks were incubated under the same conditions and after 2 days the total contents transferred to a 100-litre mechanically agitated vessel containing 70 litres of the same medium,

Table 1. Cultural properties of strain of *Amycolatopsis orientalis*, ATCC 53550.

Medium	ref	Growth	Surface color	Texture	Aerial mycerium	Reverse color	Soluble pigment
Yeast extract - malt extract	5	Good	Cream to pale yellowish, 2ca, 2ea	Raised, wrinkled	None	Pale yellowish, 2ea, 2ga	Yellowish brown, 31c
Oatmeal	5	Poor to moderate	Cream, 2ca	Thin, smooth	None	Cream, 2ca	Cream, 2ca
Inorganic salts - starch	5	Poor	Colourless to cream, 2ca	Thin, smooth	None	As surface	None
Glycerol - asparagine	5	Poor to moderate	Cream, 2ca	Slightly raised, smooth to granular	None	Cream to pale yellowish, 2ca, 2ea	None
CZAPEK - sucrose	13	Poor to moderate	Cream, 2ca	Thin, smooth	None	Colourless to cream, 2ca	None
Glucose - asparagine	13	Moderate	Cream, 2ca	Slightly to moderately raised, smooth to granular	None	Cream to pale yellowish 2ca, 2ea	None
EMERSON'S	13	Good to excellent	Cream, pale yellowish to yellowish, 2ca, 2ea, 2ga	Raised, wrinkled	None	Dark yellowish, 21c, 2nc	Yellowish brown, 31c
Nutrient	13	Moderate	Pale yellowish, 2ea	Slightly raised, smooth	None	Yellowish, 2ia, 2la	None
BENNETT'S	13	Good	Cream, 2ca	Raised, smooth to wrinkled	None	Yellowish, 2ga, 2ia	Pale yellowish, 2ea
GORDON and SMITH'S tyrosine	14	Moderate	Dark yellowish, to brown, 21e, 4ng	Slightly to moderately raised, smooth but wrinkled towards end of streak	None	Brown, 3ne	Dark brown, 4pl
Calcium malate	15	Moderate	Cream, 2ca	Thin to moderately raised, smooth to granular	None	Pale yellowish, 2ea	None
Gelatin	16	Good	Yellowish, 2ga	Moderately raised, smooth but wrinkled towards edge	None	Yellowish, 2ga	None
Starch	16	Good	Yellowish, 2ga	Moderately raised smooth but wrinkled towards end of streak	None	Yellowish, 2ec, 2ic	None
Potato carrot	7	Poor to moderate	Cream, 2ca	Thin, smooth	None	Cream, 2ca	None
Tap water		Poor	Colourless to cream, 1.5ca	Thin, smooth	None	As surface	None

Table 2. Physiological and biochemical properties of culture *Amycolatopsis orientalis*, ATCC 53550.

(A) Temperature relationships

No growth	10, 37, 45°C
Good growth	20°C
Good to excellent growth	28°C

(B)

Decomposition of:		Growth in:	
Adenine	+	Lysozyme broth	−
Calcium malate	+	5% NaCl	+
Casein	+	Clearing and coagulation of:	
Cellulose	−	Skim milk	+
Hypoxanthine	+	Utilisation of:	
Tyrosine	+	Acetate	+
Xanthine	+	Benzoate	−
Hydrolysis of:		Citrate	+
Esculin	−	Dextrin	−
Hippurate	−	Lactate	+
Starch	+	Malate	+
Production of:		Mucate	−
Gelatinase	+	Oxalate	−
Nitrate reductase	+	Phenol	−
Phosphatase	+	Propionate	+
Urease	+	Pyruvate	+
Hydrogen sulfide	+	Succinate	+
Melanin	−		

Table 3. Acid production from (and utilisation of) carbohydrates of strain *Amycolatopsis orientalis*, ATCC 53550.

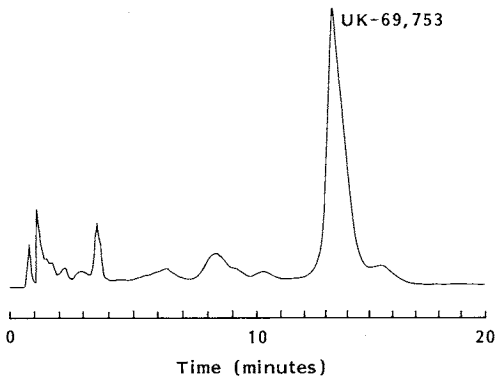
Glucose	+(+)	Glycerol	+(+)
Arabinose	+(+)	Lactose	−(−)
Fructose	+(+)	Maltose	−(+)
Inositol	−(−)	Mannose	+(+)
Mannitol	+(+)	Melezitose	−(−)
Raffinose	+(+)[a]	Melibiose	−(−)
Rhamnose	+(+)	α-Methyl-D-glucoside	−(−)
Sucrose	−(+)	Ribose	+(+)
Xylose	+(+)	Salicin	−(+)
Adonitol	+(+)	Sorbitol	+(+)
Cellobiose	+(+)	Sorbose	−(−)
Dulcitol	−(+)	Starch	+(+)
Erythritol	+(+)	Trehalose	+(+)
Galactose	+(+)		

[a] The culture did not use raffinose when the method of SHIRLING and GOTTLIEB[5] was used.

and incubated at 28°C for a further 2 days. This third stage seed culture was then inoculated into a 2,000-litre fermenter containing 1,200 litres of production medium consisting of glucose monohydrate (Cerelose) 12 kg, corn starch 12 kg, soya flour 12 kg, distillers solubles 6 kg (Scotaferm), NaCl 6 kg, $CoCl_2$ 1.2 g, Polypropylene glycol 2000 antifoam 1 kg and adjusted to pH 6.8. The fermentation ran for 7 days at 28°C, aerated at 1,200 litres/minute and agitated at 180 rpm.

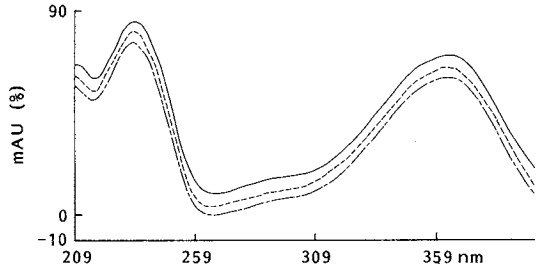
The antibiotic potency in the fermentation broth was monitored by reversed phase HPLC using a Hewlett Packard 1090A with diode array detection. Under the chromatographic conditions shown in Fig. 1 the UK-69,753 peak eluted at 13.8 minutes. UV spectra of the antibiotic were recorded on the upslope,

Fig. 1. Reverse phase chromatogram of an extract containing UK-69,753.



Column: Waters μBondapak C18 (4 × 150 mm), mobile phase: acetonitrile - 0.1 M KH_2PO_4 (35 : 65) adjusted to pH 3.5 with H_3PO_4, detection: UV 350 nm, flow: 1.5 ml/minute, temperature: 40°C.

Fig. 2. UV spectrum of UK-69,753.



	Upslope	Apex	Downslope
Spectrum (minutes)	13.4693	13.8437	14.0932
Reference (minutes)	15.2162	15.2162	15.2162
Attenuation [mAU]	300.5	405.5	263.5
Absorbance [mAU] (nm)	257.6 (235/2)	347.5 (235/2)	225.8 (235/2)

Fig. 3. Fermentation profile of *Amycolatopsis orientalis* strain N731-15.

△ UK-69,753, ○ packed mycelium volume, □ pH.

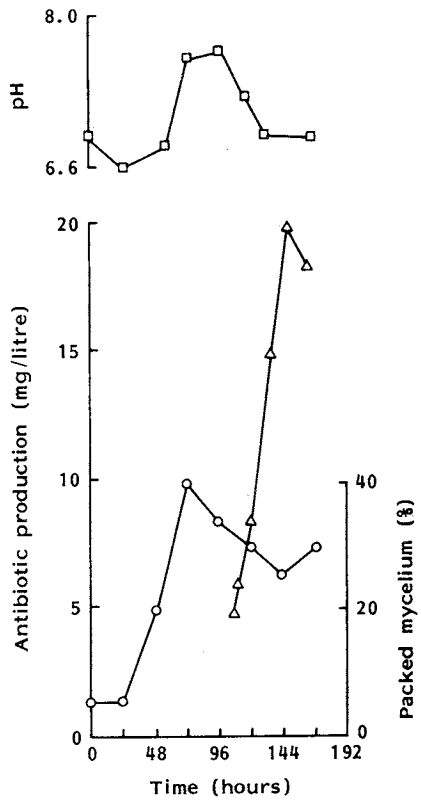


Fig. 4. Isolation and purification of UK-69,753.

Whole broth (1,200 litres)

↓ filtered

Filtrate

↓ extracted with EtOAc

Concentrated EtOAc solubles (1 litre)

↓ triturated with hexane (4 litres)

Precipitated solid (28 g)

↓ six tube counter current distribution chromatography
EtOH - H_2O (3 : 1) lower layer (2 litres) and toluene upper layer (2 litres)

Concentrated lower layer

↓ extracted with CH_2Cl_2

Semi-solid (12.2 g)

↓ dissolved in EtOAc (300 ml) and poured into hexane (700 ml)

Yellow tan precipitate (9.0 g)

↓ silica gel chromatography eluted with $CHCl_3$ - MeOH (3 : 1)

UK-69,753 (3.9 g)

apex and downslope of the chromatographic peak as shown in Fig. 2. Their synchronous nature is a good indication of the high purity of the UK-69,753 peak, assuming there is no peak coelution with identical UV chromophores. The fermentation profile is shown in Fig. 3 where the antibiotic titre peaked at 152 hours.

Isolation and Purification

The isolation procedure for the UK-69,753 complex is shown in Fig. 4.

The whole broth was filtered, and the filtrate extracted with ethyl acetate. The organic phase was then concentrated to an oily residue, estimated by HPLC to contain 20 g of UK-69,753. The residue was triturated with hexane, and the precipitate collected by filtration. The solid was subjected to a six-tube counter current distribution, using an ethanol - water mix as the lower layer and toluene as the upper layer. The antibiotic concentrated in the first three lower layers which were combined and the ethanol removed under reduced pressure. The resulting aqueous phase was extracted three times with methylene chloride, and then all organic layers combined, dried over sodium sulfate and concentrated to give a semi-solid. The residue was dissolved in ethyl acetate and poured slowly into rapidly stirred hexane. The resulting precipitate was filtered and vacuum dried to give a yellow-tan solid. The solid was chromatographed on an open silica gel support, and eluted with a chloroform - methanol gradient. Each fraction was analysed by TLC; the active fractions were combined to yield 3.9 g of UK-69,753 as an amorphous yellow solid. The physico-chemical characterisation and structural elucidation is described in the following paper.[12)]

Biological Activity

Antibiotic UK-69,753 inhibits the growth of a number of microorganisms *in vitro*. The accompanying paper[12)] indicates that the antibiotic is especially useful in the control of the anaerobic bacteria *Clostridium difficile* and the swine pathogen *Treponema hyodysenteriae*.

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