ASK-753, A NEW IRON-CONTAINING ANTIBIOTIC

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A new iron-containing peptide which inhibits the growth of Gram-positive and some Gram-negative bacteria was isolated from the broth of *Streptomyces* AS-K-753. The antibiotic was obtained as light buff plates. On acid hydrolysis eight amino acids, three organic acids and one keto acid could be detected. ASK-753 is similar in many properties to the sideromycin but differs from it in certain crucial characteristics.

In a course of screening studies, a new iron-containing polypeptide was obtained from the culture broth of *Streptomyces* AS-K-753 which had been isolated from the soil of U. A. R. The antibiotic is mainly active against Gram-positive bacteria and to a lesser extent against Gram-negative organisms and some non-filamentous fungi. ASK-753 is scarcely soluble in water and insoluble in petroleum ether but freely soluble in most organic solvents.

In the present paper the characteristics of the producing organism, the isolation procedure, physical and chemical properties and some biological activities of the antibiotic are given.

Antibiotic-Producing Organism

The growth characteristics of *Streptomyces* AS-K-753 were examined by allowing the organism to grow on a variety of media at 28°C for 14 days (Table 1). The ability of the organism to utilize different carbon sources was also investigated (Table 2). Microscopic examination of cultures grown on starch-nitrate medium revealed long open lateral spiral sporophores (Plate 1). The aerial hyphae are velvety grey originating from pale grey monopodially branched substrate hyphae. The electron

Plate 1. Sporophores of *Streptomyces* AS-K-753 (×600×⅔)

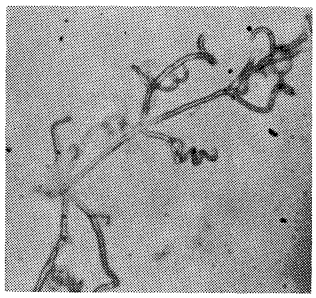


Plate 2. Electron micrograph of spores of *Streptomyces* AS-K-753 (×16,000×⅔)

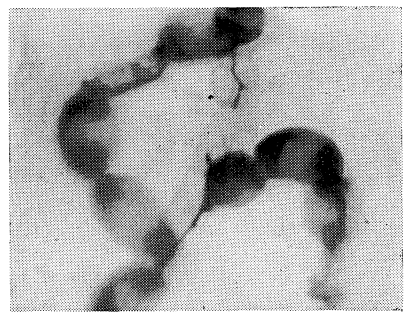


Table 1. Culture characteristics of strain AS-K-753

Medium	Characteristics	Medium	Characteristics
Nutrient agar	G. good A. white, velvety Sub. creamy S. none	Starch nitrate	G. good A. light grey, velvety Sub. pale grey S. none
Glucose nitrate	G. good A. light grey Sub. pale grey S. none	Milk	G. good, whitish yellow, coagulation A. whitish yellow Sub. moderate peptonization S. straw yellow
Glycerol asparagine	G. good A. pallid grey, velvety Sub. light grey S. none	Nitrate reduction	G. whitish grey, reduction occurs slowly, medium turns yellow
Potato	G. good A. pale grey Sub. deep grey S. none	Gelation stab	G. whitish colonies A. light brown Gelatin liquefaction, rapid
Glucose asparagine	G. good A. pallid grey, velvety Sub. light grey S. none	Cellulose	G. moderate A. light grey Sub. colourless S. none
H_2S formation	G. good Sub. pallid grey A. Pallid grey S. none	Melanin formation	Negative

G : growth; A : aerial mycelium; Sub : substrate mycelium; S : Soluble pigment.

Table 2. Carbon source utilization

Carbon source	Utilization	Carbon source	Utilization
D-Glucose	++	D-Mannitol	++
D-Fructose	+	L-Arabinose	+++
D-Maltose	++	Sucrose	+++
D-Xylose	+++	Rhamnose	+
D-Sorbitol	+	Glycerol	+++
D-Raffinose	+	Inulin	+
D-Lactose	+	Starch	++
D-Galactose	+	Sodium citrate	+

where − : no growth; + : feeble growth; ++ : moderate growth; +++ : vigorous growth

micrograph showed oval spores with smooth surface and a middle pale strip between two darker terminal zones (Plate 2).

Organisms which resemble *Streptomyces* AS-K-753 in having grey aerial mycelium and which give negative melanin test are; *S. parvullus*, *S. intermedius*, *S. craterifer* and *S. cellulosae*. WAKSMAN[1] considered these organisms as members of the cinereus series. None of these organisms possesses long lateral spiral sporophores. *Streptomyces*

parvullus and *S. intermedius* secrete soluble pigments on glucose-asparagine and potato media, while AS-K-753 fails.

Fermentation and Isolation of Antibiotic ASK-753

Streptomyces AS-K-753 grows readily on a medium of the following composition in g/liter: glucose 40, urea 0.4, K_2HPO_4 0.8, KCl 0.6, $MgSO_4 \cdot 7H_2O$ 0.6, $FeSO_4 \cdot 5H_2O$ 0.0008 at pH 7.5 before sterilization. Maximum yield of the antibiotic was attained after 48~72 hours. The broth was freed from mycelium and then extracted with chloroform at pH 7.5~8.0. The extract was repeatedly washed with phosphate buffer of pH 4.0. These washings removed most of the pigments and other impurities. The chloroform solution of the antibiotic was evaporated to dryness to yield a brownish buff powder. The crude antibiotic was then redissolved in chloroform-ethanol mixture (99:1) and the solution was allowed to pass through a Sephadex LH-20 column. The antibiotic moves as a buff coloured zone which was separated and concentrated to yield the antibiotic as light buff plates.

Physical and Chemical Properties of Antibiotic ASK-753

The antibiotic crystalizes as light buff plates of no characteristic odour. On gradual heating the colour changes to brown at 80°C then to deep brown at 100°C with charring at 120°C. ASK-753 is freely soluble in chloroform, acetone, diethyl ether, ethyl and butyl alcohols; hardly soluble in ethyl and butyl acetates and water and insoluble in petroleum ether.

The ultraviolet absorption spectrum of the antibioticc (Fig. 1) in neutral ethanol exhibits a peak at 270 mμ

Fig. 1. Ultraviolet spectrum of antibiotic ASK-753

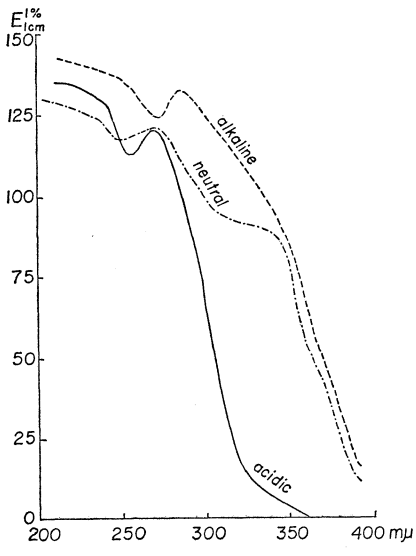


Fig. 2. Infrared spectrum of antibiotic ASK-753 pelletted in KBr

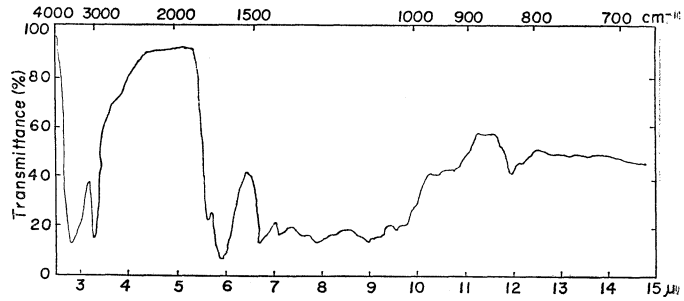
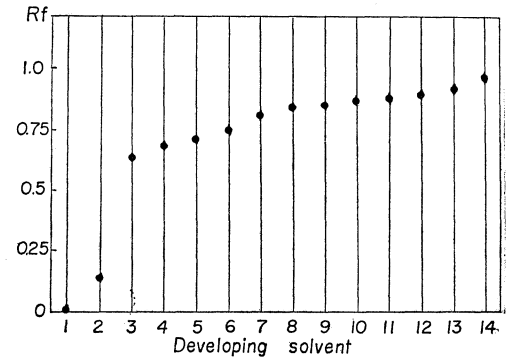


Fig. 3. Behaviour of ASK-753 when chromatographed with various developing solvents



($E^{1\%}_{1cm}$ 120.7); in acidic ethanol at 270 mμ ($E^{1\%}_{1cm}$ 120) and in alkaline ethanol at 287 mμ ($E^{1\%}_{1cm}$ 135.2). The infrared spectrum of ASK-753 pelletted in KBr showed characteristic bands at 3500, 1670, 1480 and 837 cm^{-1} (Fig. 2).

Migration of ASK-753 by descending paper chromatography using Whatman No. 1 filter sheets and different developing solvents is illustrated in Fig. 3. The antibiotic resembles the fast moving type of sideromycins[2] when developed with butanol-acetic acid-water (4:1:5). Location of the zone containing the antibiotic was performed bioautographically using *Bacillus subtilis* as the test organism. One definite inhibition zone was always observed. Another group of strips was sprayed with dilute potassium permanganate solution where no zones other than those of the antibiotic could be observed.

Elemental analysis :

Found C 54.64, H 6.98, N 6.92,
O 28.96, Fe 2.5 %

The behaviour of the antibiotic towards different chemical tests is shown in Table 3. On hydrolysing ASK-753 in 6 N HCl at 105°C for 24 hours eight amino acids and four organic acids were liberated. By the descending paper chromatographic technique the following hydrolytic products could be identified : lysine, glutamic acid, aspartic acid, gylcine, alanine and leucine. The two unidentified amino acids possessed Rf values 0.32 and 0.38 when chromatographed on Whatman No. 1 paper strips using butanol-acetic acid-water (4:1:5) as the developing solvent. The organic acids which were detected in the hydrolysate were gluconic, citric, fumaric and α-ketoglutaric acids. The content of α-ketoglutaric acid was comparatively minute.

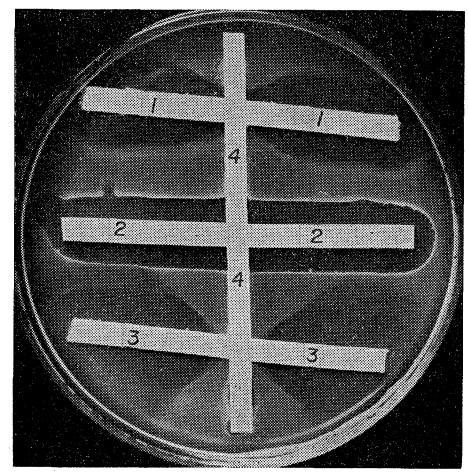
Table 3. Behaviour of ASK-753 towards different chemical tests

Chemical test	Reult
Alkaline $KMnO_4$	Reduction on cold
Acidic $KMnO_4$	Reduction on cold
Molisch's test	Negative
Fehling solution	Negative
Ninhydrin	Negative
Biuret	Negative
Millon's test	Negative
Sakaguchi's test	Negative
Ferric chloride solution	Negative
Potassium thiocyanate	Red colour after ignition and dissolved in nitric acid
Potassium ferrocyanide	Blue colour after ignition and dissolved in nitric acid
Iodine solution	No absorption of iodine
Nitration	Negative

Plate 3. Antimicrobial effects of ferrimycin; antibiotics ASK-753 and 22765 in presence of ferrioxamine B

Strip 1 loaded with ferrimycin*
" 2 " " ASK-753
" 3 " " 22765*
" 4 " " ferrioxamine B*

* These substances were kindly offered by Dr. H. Bickel, assistant manager, Ciba Ltd., Switzerland.



Biological Properties of ASK-753

Antimicrobial activity: The antimicrobial activity of ASK-753 against a variety of microbes was studied (Table 4). The results demonstrate the potent inhibitory activity against *Corynebacterium*

Table 4. Antimicrobial spectrum of ASK-753

Test organism	M. I. C. mcg/ml	Test organism	M. I. C. mcg/ml
Bacillus subtilis 24 G	0.75	*Salmonella typhosa* NRRL B-573	25.0
Bacillus subtilis 19 T	0.75	*Salmonella typhosa*	25.0
Bacillus subtilis D_{161} (chlortetracycline R.)	0.75	*Salmonella paratyphi* A-2 : a	>100
Bacillus subtilis AA	1.5	*Salmonella enteritidis* Univ. Ill.	>100
Bacillus subtilis ICC	1.5	*Escherichia coli* 0127 : B_8; H-VCNCTC 9709	3.12
Bacillus subtilis D_{161} (novobiocin R.)	1.5		
Bacillus subtilis D_{161} (chloramphenicol R.)	1.5	*Escherichia coli* 0127 : B_8 MJ 50	12.5
Bacillus subtilis D_{161} (streptomycin R.)	1.5	*Escherichia coli* N 27405	12.5
Bacillus subtilis D_{161} (staphylomycin R.)	1.5	*Escherichia coli* D_{165}	12.5
Bacillus subtilis NRRL-B-543	3.12	*Escherichia coli* NRRL B-210	50.0
Bacillus subtilis D_{161} (spiramycin R.)	3.12	*Klebsiella pneumoniae* NRRL B-36	6.25
Bacillus mycoides (U. S. S. R.)	12.50	*Klebsieilla pneumoniae* 0-1-K3NCTC5056	25.0
Bacillus cereus D_{166} (oxytetracycline R.)	25.0		
Bacillus cereus D_{166} (kanamycin R.)	25.0	*Klebsieilla pneumoniae* NRRL B-117	50.0
Bacillus cereus NRRL B-569	50.0	*Klebsieilla pneumoniae* 1231-67-CDC	50.0
Bacillus diphtheroid	>100	*Shigella boydii* 22854-61-CDC	6.25
Staphylococcus aureus D_6 (paromycin R.)	0.35	*Shigella equirulis* H-33	25.0
Staphylococcus aureus A_{55}	0.75	*Proteus vulgaris* Pr. 1 CDC	1.5
Staphylococcus aureus D_{66} (PKAM R.)	0.75	*Proteus mirabilis* Su I_2	12.5
Staphylococcus aureus D_6(oleandomycin R.)	1.5	*Proteus mirabilis* H-3	12.5
Staphylococcus aureus D_6(streptomycin R.)	1.5	*Proteus rettgeri* SuI_9	50.0
Staphylococcus aureus NRRL B-313	3.12	*Pseudomonas aeruginosa* D_{129}	0.37
Staphylococcus rosea	12.5	*Pseudomonas aeruginosa* SuI 14	>100
Staphylococcus aureus FDA 209 P	25.0	*Pseudomonas aeruginosa* ATCC 14502	>100
Staphylococcus aureus I-42/3	50.0	*Pseudomonas aeruginosa* 9027	>100
Corynebacterium hoffmanii	0.31	*Haemophilus influenza* A-733	0.75
Corynebacterium michiganense NRRL B-33	3.12	*Enterobacter aerogenes* 659-66-CDC	>100
Corynebacterium minutissimum UP 54	>100	*Saccharomyces cerevisiae* NKRL Y-567	3.12
Salmonella paratyphi C-6, 7 : C	12.5	*Candida albicans* NRRL Y-477	6.25
Salmonella paratyphi B-4, 6 : b	25.0	*Aspergillus niger*	>100

M. I. C. = Minimum inhibitory concentration R = Resistant

hoffmanii, *Pseudomonas aeruginosa* D_{129} and most Gram-positive bacilli and cocci. Limited activity was observed against Gram-negative bacteria and *Saccharomyces cerevisiae*. ASK-753 is active against *Staphylococcus aureus* resistant to streptomycin, chloramphenicol, oxytetracycline, novobiocin, chlortetracycline and spiramycin.

It is of particular interest that the antibacterial effect of ASK-753 is not antagonized by ferrioxamine B, a sideramine (Plate 3). Ferrioxamine B failed also to antagonize the antimicrobial effects of the antibiotic ferramido chloromycin (FACM)[7]. Meanwhile the same sample of sideramine did antagonize the antimicrobial effect of ferrimycin and antibiotic No. 22765 (Plate 3).

In vivo test: Tests were done with mice infected intraperitoneally with *Staph. aureus* A 321 (a penicillin sensitive strain); 5 mice were used for each series. Protection tests were made by giving two intraperitoneal injections both on the day of infection. The dose reported therefore is twice the amount used for each injection. The CD_{50} found was 10 mg/kg body weight.

Toxicity tests: The LD_{50} for Swiss mice was found to be 58 mg/kg body weight (95 % reliability range 43~79 mg/kg) by intraperitoneal injection.

Discussion

Morphological and culture characteristics of *Streptomyces* AS-K-753 differentiate this organism from *S. craterifer*, *S. intermedius*, *S. parvullus* and *S. cellulosae*. Nevertheless, all the strains fail to give positive melanin test and possess different shades of grey aerial hyphae.

Antibiotic ASK-753 is rather unique in having citric, fumaric, gluconic and α-ketoglutaric acids in addition to amino acids in its molecule. The information collected from the elemental analysis, U. V. and I. R. spectra, its behaviour towards different chemical tests and its acid hydrolysis indicate clearly its peptide nature and uniqueness among the iron-containing peptide antibiotics.

ASK-753 resembles members of the sideromycin[2~6] antibiotic group in being a polypeptide containing iron. Nevertheless, it differs from this group in having a pale buff colour and being hardly soluble in water. Its antimicrobial activity is not antagonized by the sideramine and ferrioxamine B. For this reason ASK-753 is not considered to be a true sideromycin and could tentatively be termed 'Pseudosideromycin'. Ferramido chloromycin (FACM)[7] is also rather insoluble in water and its effect is not abolished by sideramines.

ASK-753 resembles the fast-moving group[2] of sideromycins on paper chromatograms when developed with butanol - acetic acid - water (4 : 1 : 5), but differs in its scope of antimicrobial activity.

Ackonwledgment

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References

1) WAKSMAN, S. A. : The actinomycetes. Vol. 2, Chapt. 8, Description of species of *Streptomyces*. pp. 165~292, The Williams & Wilkins Co., 1961
2) BICKEL, H.; E. GÄUMANN, G. NUSSBERGER, P. REUSSER, E. VISCHER, W. VOSER, A. WETTSTEIN & H. ZÄHNER : Stoffwechselprodukte von Actinomyceten. Über die Isolierung und Charakterisierung der Ferrimycine A_1 und A_2, neuer Antibiotika der Sideromycin-Gruppe. Helvet. Chim. Acta 43 : 2105~2118, 1960
3) UMEZAWA, H. : Recent advances in chemistry and biochemistry of antibiotics. Inst. Microb. Chem., Tokyo 1964
4) KUEHL, F. A., Jr.; M. N. BISHOP, L. CHAIET & K. FOLKERS : Isolation and some chemical properties of grisein. J. Am. Chem. Soc. 73 : 1770~1773, 1951
5) GAUSE, G. F. : Recent studies on albomycin, a new antibiotic. Brit. Med. J. 1955-2 : 1177~1179, 1955
6) ZÄHNER, H.; R. HÜTTER & E. BACHMANN : Stoffwechselprodukte von Actinomyceten. XXIII. Zur Kenntnis der Sideromycinwirkung. Arch. Mikrobiol. 36 : 325~349, 1960
7) SHIMI, I. R. & S. SHOUKRY : Ferramido chloromycin, a new antibiotic. J. Antibiotics, Ser. A 19 : 110~114, 1966