YEMENIMYCIN, A NEW ANTIBIOTIC

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A new antibiotic which inhibits the growth of Gram-positive bacteria and fungi was isolated from the broth and mycelial pellets of *Streptomyces* AS-Y-52. The antibiotic was obtained as pale buff minute needles with a yellow tinge which on acid hydrolysis gave eight ninhydrin-positive products including arginine, aspartic acid, glutamic acid, alanine, phenylalanine and isoleucine. Characteristics of the organism, isolation of the antibiotic as well as its physical, chemical and biological properties are given.

During our screening program devoted to isolate new antibiotics from cultures of microorganisms obtained from the soil of the Middle East area a potent antifungal and anti-Gram-positive antibiotic, yemenimycin, could be isolated. The antibiotic is produced in the culture filtrate and mycelial pellets of *Streptomyces* AS-Y-52 isolated from a soil sample of the northern part of Yemen lands. Yemenimycin is a polypeptide which is soluble in most organic solvents, but hardly soluble in water and petroleum ether.

Producing Organism

Streptomyces AS-Y-52 was grown on different media at 28°C for 14 days and the culture characteristics were recorded (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 straight to wavy sporophores (Plate 1), while the electron micrograph magnified the sporophores to be oval spores with smooth surfaces (Plate 2).

Plate 1. Sporophores of *Streptomyces* AS-Y-52 (×600×1.5)

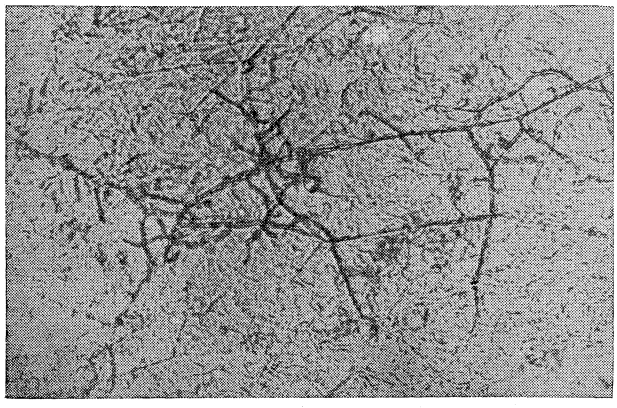

Plate 2. Electron micrograph of spores of *Streptomyces* AS-Y-52 (×20,000×1.5)

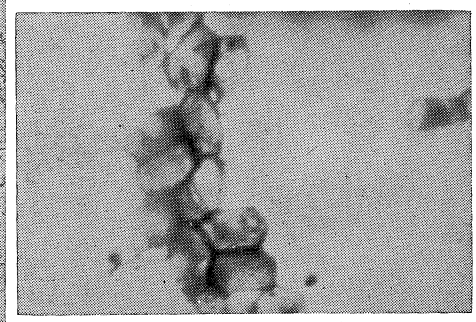

Table 1. Culture characteristics of strain AS-Y-52

Medium	Characteristics	Medium	Characteristics
Nutrient agar	G. moderate A. poor Sub. pale grey S. none	Milk	G. good A. pallid grey Good coagulation and peptonization at pH 7
Glucose-nitrate	G. moderate A. white Sub. pale brown S. none	Nitrate reduction	G. good A. pallid grey S. none Reduction after 3 days
Starch-nitrate	G. moderate A. white Sub. pale brown S. pallid brown	Gelatin stab	G. good A. pallid grey Sub. dark brown S. none Gelatin liquefaction good
Glucose-asparagine	G. poor A. none Sub. white S. none	H_2S	G. poor A. none Sub. pale brown S. none
Glycerol-asparagine	G. moderate A. pallid grey Sub. pale brown S. none	Potato	S. good A. white Sub. brown S. brown
Cellulose	No growth	Melanin formation	Negative

G : growth, A : aerial mycelium, Sub : substrate mycelium, S : soluble pigment.

Table 2. Utilization of carbon sources

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

− : no growth, + : feeble growth, ++ : moderate growth, +++ : strong growth.

The organism is characterized by its white to light grey aerial mycelium and by its negative melanin test. A faint brownish pigment is occasionally produced on natural media. According to WAKSMAN's classification system[1], this organism belongs to the albus series. Among the organisms that belong to this series and show similarity to AS-Y-52 are *S. calvus*, *S. niveus* and *S. mirabilis*. The first two organisms differ from AS-Y-52 in being able to utilize xylose and arabinose, while AS-Y-52 failed. *S. niveus* is unable to reduce nitrate, while AS-Y-52 could. *S. mirabilis* is differentiated from AS-Y-52 by secreting the dark brown pigment in gelatin stab.

Production and Isolation of Yemenimycin

Streptomyces AS-Y-52 thrives at 28°C on the following medium: (g/liter) soluble starch 20, $NaNO_3$ 2.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, $FeSO_4 \cdot 5H_2O$ 0.000.5, at

pH 7. Maximum yields of the antibiotic could be obtained after 6 days of incubation in shaken cultures at 200 r.p.m. The broth was freed from mycelial pellets and then extracted with a mixture of chloroform - ethylacetate (1 : 1) at pH 7.0~7.5. The extract of the broth was repeatedly washed with carbonate-bicarbonate buffer at pH 10.0 in order to remove pigments and other inactive impurities. The solvent layer was then freed from traces of buffer by shaking with distilled water. The organic phase containing the active substance was then evaporated to dryness under reduced pressure to yield a dark brownish residue which possessed a potent antimicrobial activity. The crude yemenimycin when dissolved in absolute methanol, left an inactive dark brown precipitate. For further purification the antibiotic was dissolved in dry methanol which wes then passd through a short column of active charcoal (Darco G 60) that adsorbed the residual pigments and a small portion of the antibiotic. The methanolic solution, if left to evaporate solwly, would yield pale yellowish buff minute needles of yemenimycin. The mycelial pellets were liberated from excess liquid, and suspended in acetone. The suspension was shaken for 6 hours. The acetone extract, which showed considerable activity, was evaporated to dryness in vacuum and the residue was then extracted with dry methanol. Further purification was carried out as described above.

Physical and Chemical Properties of Yemenimycin

Highly purified yemenimycin is formed of pale buff minute needles with a yellowish tinge which on gradual heating darkens at 194°C and finally melts at 212~214°C. The antibiotic is freely soluble in chloroform, acetone, ethyl and butyl acetates, moderately soluble in methyl, ethyl and butyl alcohols, and hardly soluble in water and petroleum ether.

The ultraviolet absorption spectrum of the antibiotic is shown in Fig. 1. The ethanolic solution of the antibiotic shows two absorption maxima, at 230 mμ ($E^{1\%}_{1cm}$ 449) and at 314 mμ ($E^{1\%}_{1cm}$ 626). A shift to 235 mμ ($E^{1\%}_{1cm}$ 440) and 356 mμ ($E^{1\%}_{1cm}$ 558) recorded in the

Fig. 1. Ultraviolet spectrum of yemenimycin in ethanol.

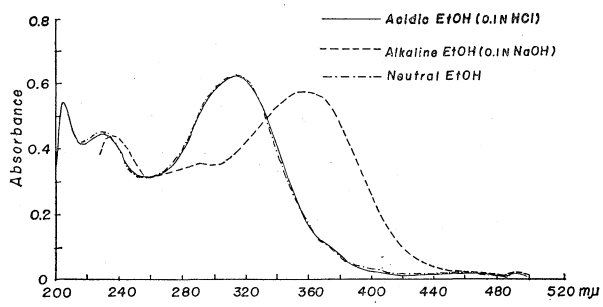

Fig. 2. Infrared spectrum of yemenimycin pelletted in KBr.

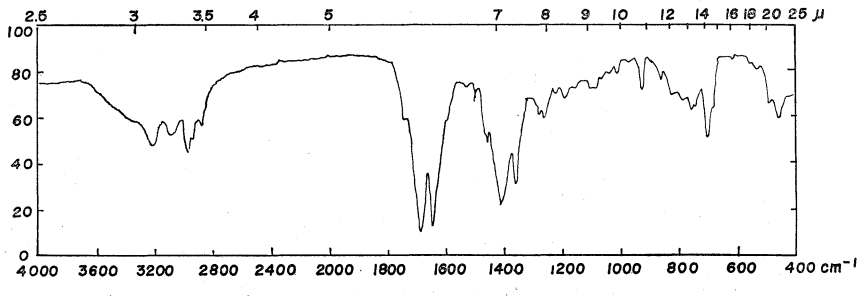

Fig. 3. Migration of yemenimycin with different developing solvents.

1. Petroleum ether, b.p. 40~60℃
2. Petroleum ether, b.p. 100~120℃
3. Distilled water
4. *n*-Butanol - water (1 : 1)
5. Methanol
6. *n*-Butanol - acetic acid - water (4 : 1 : 5)
7. Ethyl acetate - water (1 : 1)
8. Chloroform saturated with water
9. Ethyl acetate-petroleum ether
10. Ethanol
11. Chloroform
12. Ethyl acetate
13. Acetone

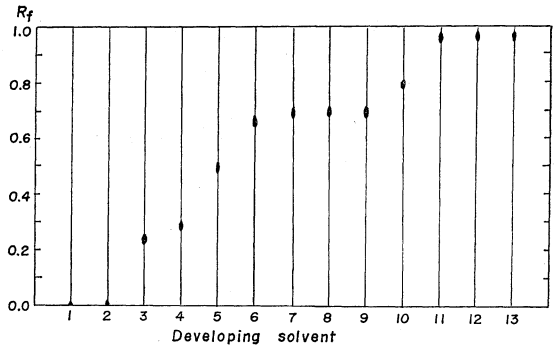

Table 3. Behavior of yemenimycin towards different chemical tests

Chemical tests	Results
1. Alkaline $KMnO_4$	Reduction on heating
2. Acidic $KMnO_4$	Reduction on heating
3. MOLISCH's test	Negative
4. FEHLING solution	Negative
5. Ninhydrin reagent	Negative
6. Biuret test	Negative
7. MILLON's test	Positive
8. SAKAGUCHI's test	Negative
9. Ferric chloride solution	Negative
10. Iodine solution	No absorption of iodine
11. Nitration test	Positive
12. Potassium thiocyanate	Negative
13. Potassium ferrocyanide	Negative
14. Acid hydrolysate +diazotized anthranilic acid	Positive color test

alkaline alcohol. The infrared spectrum of yemenimycin pelleted in KBr (Fig. 2) exhibited characteristic bands at 3200, 3100, 2980, 2950, 2890, 1690, 1645, 1500, 1460, 1410, 1360, 1280, 1265, 930 and 700 wave numbers. In view of this absorption pattern the presence of the following groups in the molecule of the antibiotic is indicated : NH, OH, CH_2, CH_3, NH_2, $CONH_2$ and probably aromatic structure. The optical rotation is $[\alpha]_D^{20}$ $-268°$ in methanol.

The migration of yemenimycin on paper chromatograms, when developed by different solvents, is displayed in Fig. 3. The zone containing the antibiotic was located bioautographically using *Bacillus subtilis* NRRL-B-543 and *Penicillium chrysogenum* Q 176 as test organisms. One definite inhibition zone was always observed. Another group of developed strips were sprayed with dilute potassium permanganate solution and heated; no zone other than those of the antibiotic could be observed.

Anal. Calcd. for $C_{41}H_{43}N_4ClO_9$; C 63.85, H 5.58, N 7.28, Cl 4.61, O 18.69
Found ; C 63.76, H 5.60, N 7.27, Cl 4.61, O 18.75

The behavior of the antibiotic towards the different chemical tests is given in Table 3. Yemenimycin reduced both alkaline and acidic potassium permanganate on heating, while it failed to give positive reaction with MOLISCH's and FEHLING's tests indicating the absence of reactive reducing sugar moieties. That both ninhydrin reagent and biuret's test gave negative results may indicate the cyclic nature of this polypeptide. The existence of aromatic structure in the molecule of yemenimycin is confirmed by the positive nitration test. Acid hydrolysis of yemenimycin with 6 N HCl at 105°C for 24 hours liberated arginine, aspartic acid, glutamic acid, alanine, phenylalanine, isoleucine and two other ninhydrin-positive hydrolytic products. These hydrolytic products were identified by paper chromatographic techniques (one and two

dimensional) as well as by thin-layer chromatography using different adsorbants. Samples of the acid hydrolysate were always situated in line with and in between two lateral spots of each authentic amino acid.

Biological Properties of Yemenimycin

Antimicrobial activities :

Yemenimycin exerts notable activities against Gram-positive bacteria. The minimum inhibitory concentrations (MIC's) against the test organisms tried, ranged from 0.006 to 0.78 mcg/ml. *Staphylococcus aureus*, *Micrococci*, *Bacillus cereus* and *Staphylococcus pyogenes* are highly sensitive to the antibiotic. Activities of yemenimycin against representatives of Gram-negative bacteria were, however, very weak. Feeble

Table 4. Antibacterial activities of yemenimycin

Test bacteria	MIC mcg/ml	Test bacteria	MIC mcg/ml
Bacillus cereus	0.006	*Proteus mirabilis* SU 12	6.25
Bacillus subtilis ATCC 9524	0.78	*Proteus morganii* 1166	6.25
Corynebacterium minutissimum UP 54	0.19	*Pseudomonas aeruginosa* ATCC 9027	100
Micrococcus lysodeikticus	0.006	*Pseudomonas aeruginosa* ATCC 14502	100
Micrococcus pyogenes var. *albus*	0.006	*Pseudomonas aeruginosa* UL 10	100
Sarcina lutea ATCC 9341	0.19	*Pseudomonas aeruginosa* SUI 14	100
Staphylococcus aureus 209 P	0.006	*Salmonella choleraesuis*	25
〃 〃 4213	0.006	〃 *derby*	100
〃 〃 Rose	0.012	〃 *enteritidis*	100
〃 〃 Smith	0.006	〃 *indiana* (white)	100
〃 〃 speier	0.006	〃 *pullorum*	12.5
Streptococcus pyogenes	0.006	〃 *typhimurium*	50
Citrobacter freundii 532-57	100	*Serratia marcescens* 252-67	100
Enterobacter aerogenes 659-66	50	*Serratia marcescens*	100
〃 *cloacae* 5680	100	*Shigella boydii* 2,285-61	12.5
Escherichia coli MJ 51	12.5	〃 *flexneri* 26,1794-65	25
〃 〃 MJ 50	100	〃 *sonnei* SUI 21	25
〃 〃 N 27405	50	*Klebsiella ozaenoe* CDC	100
Proteus rettgeri SUI 9	100	〃 *pneumoniae* CDC 3	100
Proteus vulgaris Pr-1 CDC	12.5	〃 〃	100

The MIC were determined by the serial agar dilution method.

Table 5. Antifungal activities of yemenimycin

Test fungi	MIC mcg/ml	Test fungi	MIC mcg/ml
Microsporum audouini ATCC 9079	0.012	*Candida albicans* ATCC 10231	0.095
Microsporum canis ATCC 10241	0.024	*Candida albicans* NRRL 400	0.012
Microsporum cookei SUI 1127	0.39	*Candida albicans* NRRL 477	0.012
Keratinomyces ajelloi SUI 1123	1.56	*Alternaria solani*	0.78
Trichophyton mentagrophytes (gypseum) ATCC 9129	0.012	*Rhizoctonia solani*	0.78
Trichophyton mentagrophytes (interdigitale) ATCC 9972	0.012	*Penicillium chrysogenum* NRRL Q 176	1.56
Trichophyton mentagrophytes (asteroides) ATCC 8757	0.012	*Fusarium oxysporum*	0.78
		Fusarium lycopersici	0.39
Trichophyton asteroides (Japan)	0.012	*Botrytis allii*	1.56
Trichophyton rubrum	0.095		

The MIC were determined by the serial agar dilution method.

activity was recorded against *Proteus*, *Salmonella* and *Shigella* species. These results are shown in Table 4.

Yemenimycin is notably strong against fungi. The different strains of *Candida albicans*, *Trichophyton* and *Microsporum* showed exceptionally high sensitivity to the antibiotic. The most resistant organism to the antibiotic was *Keratinomyces ajeltoi* which was inhibited at a concentration of 1.56 mcg/ml. The results of the antifungal properties of yemenimycin is given in Table 5.

Acute toxicity tests :

Limited acute toxicity tests for yemenimycin were carried out in Swiss mice. Each group of five animals received single interperitoneal injections of varying amounts of the antibiotic covering a range of 0.78~50 mg/kg. The LD_{50} was calculated on the third and seventh day post antibiotic administration. The results obtained are given in Table 6. At the third day post antibiotic administration there was 100 % mortality in all groups receiving down to 6.25 mg/kg. Delayed deaths were noted in groups which received 3.12 and 1.56 mg/kg between the third and seventh days of observation.

Table 6. Acute toxicity of yemenimycin

Single i. p dose (mg/kg)	Percent death after	
	3 days	7 days
50	100	100
25	100	100
12.5	100	100
6.25	100	100
3.12	40	100
1.56	20	100
0.78	20	20
ALD_{50} (mg/kg)	3.5	1.1

A single daily application of 0.00001 % of aqueous alcoholic solution of yemenimycin to superficial wounds of Swiss mice for 14 consecutive days exhibited no obvious toxic symptoms. Subsequent to stopping of the application, the animals lived for several months without toxicity symptoms.

Discussion

Morphological and culture characteristics of *Streptomyces* AS-Y-52 place this organism in the albus series. Its physiological and biochemical properties differentiate it from some species of this series which show resemblance to it such as *S. calvus*, *S. niveus* and *S. mirabilis*.

The information collected from the elemental analysis of yemenimycin, its physical and chemical properties as well as its acid hydrolysis suggest the polypeptide nature of the antibiotic. According to UMEZAWA[3], yemenimycin could be classified as a member of the group of antibiotics soluble in organic solvents. Nevertheless, yemenimycin possesses properties which distinguish it from other antibiotics of antifungal and anti-Gram-positive antibiotics hitherto known. Considering the scope of their antimicrobial spectra, the following non-polyene antibiotics exhibit some resemblance to yemenimycin : eulicin[3], seligocidin[4], flavofungin[5], alboverticillin[6], cellostatin[7], xanthicin[8], blasticidin S[9], musarin[10], lustericin[11], hygrostatin[12], anthelmycin[13], PA 132[14], FH 3582[15], SQ 15852[16] and pyrrolnitrin[17]. These products differ cruciably from yemenimycin in their chemical nature, physical properties and biological activities.

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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) UMEZAWA, H. : Recent advances in chemistry and biochemistry of antibiotics. Inst. Microb. Chem., Tokyo, 1964
3) CHARNEY, J.; R. A. MACHLOWITZ, F. J. MCCARTHY, G. A. RUTKOWSKI, A. A. TYTELL & W. P. FISHER : Eulicin, a new antifungal agent. Antibiot. Ann. 1955/1956 : 228~230, 1956
4) NAKAMURA, S,; K. MAEDA, Y. OKAMI & H. UMEZAWA : A new antifungal substance of *Streptomyces seligocidin*. J. Antibiotics, Ser. A 7 : 57, 1954
5) URI, J. & I. BÉKÉSI : Flavofungin, a new crystalline antifungal antibiotic; Origin and biological properties. Nature 181 : 908, 1958
6) MAEDA, K.; S. KONDO, K. OHI, H. KONDO, E. LIN WANG, Y. OSATO & H. UMEZAWA : A new antibiotic, alboverticillin. J. Antibiotics, Ser. A 11 : 30~31, 1958
7) HAMADA, S. : A study of a new antitumor substance, cellostatin. I. On the isolation and some properties of cellostatin. Tohoku J. Exp. Med. 67 : 173~179, 1958
8) SEKIZAWA, Y. & K. MIWA : An antibiotic *Streptomyces* No. 689 strain. II. Properties of the antifungal antibiotic, xanthicin. J. Agr. Chem. Soc. Japan 30 : 471~474, 1956
9) TAKEUCHI, S.; K. HIRAYAMA, K. UEDA, H. SAKAI & H. YONEHARA : Blasticidin S, a new antibiotic. J. Antibiotics, Ser. A 11 : 1~5, 1958
10) ARNSTEIN, H. R. V.; A. H. COOK & M. S. LACEY : The inhibition of *Fusarium oxysporum* var. *cubense* by musarin, an antibiotic produced by MEREDITH's Actinomycete. J. Gen. Microbiol. 2 : 111~122, 1948
11) SHIBATA, M.; K. NAKAZAWA, M. INOUE, J. TERUMICHI & A. MIYAKE : A new antibiotic, lustericin, produced by a *Streptomyces* No. 10400. Ann. Rep. Takeda Research Lab. 17 : 19~22, 1958
12) KOJO, K.; K. SHIMIZU, H. SAKAI, M. MIKATA & T. FUJISAWA : Hydrostatin, a new antibiotic. (in Japanese) Yakugaku Kenkyu 30 : 654~664, 1958
13) HAMILL, R. L. & M. M. HOEHN : Anthelmycin, a new antibiotic with anthelmintic properties. J. Antibiotics, Ser. A 17 : 100~103, 1964
14) KOE, B. K.; B. A. SOBIN & W. D. CELMER : PA 132, a new antibiotic. I. Isolation and chemical properties. Antibiot. Ann. 1956/1957 : 672~675, 1957
15) WALLHAUSER, K. H.; G. HUBER, G. NESEMANN, P. PRÄVE & K. ZEPL : Die Antibiotika FH 3582 A und B und ihre Identität mit Nonactin und seinen Homologen. Arzneimittel-Forschung 14 : 356~360, 1964
16) BENNETT, R. E.; S. A. BRINDLE, N. A. GIUFFRE, P. W. JACKSON, J. KOWALD, F. E. PANSY, D. PERLMAN & W. H. TREJO : Production of a novel cytotoxic agent, SQ 15,859, by *Streptomyces chrysomallus*. Antimicr. Agents & Chemoth.-1961 : 169~172, 1962
17) ARIMA, K.; H. IMANAKA, M. KOUSAKA, A. FUKUDA & G. TAMURA : Pyrrolnitrin, a new antibiotic substance, produced by *Pseudomonas*. Agr. Biol. Chem. 28 : 575~576, 1964