HODYDAMYCIN, A NEW ANTIBIOTIC

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Hodydamycin is a new antibiotic which has been isolated from *Streptomyces* AS-Y-400 obtained from the soil of Yemen. The antibiotic strongly absorbs iodine and yields five aminoacids on acid hydrolyses. It melts at 145°C and shows an ultraviolet absorption maximum at 249 mμ ($E^{1\%}_{1cm}$ 358) in neutral and acidic ethanol. Hodydamycin is strictly active against Gram-positive bacteria, possesses low toxicity and markedly high serum binding property.

In a continuing search to appraise members of *Stretomyces* isolated from the soils of the Middle East area as producers of antimicrobial substances an isolate No. 400 which was obtained from the soil of Yemen was examined. It secreted in its culture filtrate a new antibiotic hodydamycin. The antibiotic is freely soluble in most of the organic solvents; slightly soluble in water and rather insoluble in petroleum ether (40~60°C). This paper describes the producing organism, the culture conditions and isolation of the antibiotic as well as its chemical, physical and biological properties.

Producing Organism

The growth characteristics of *Streptomyces* AS-Y-400 were recorded when the organism was grown on a veriety of media at 28°C for 14 days[1] (Table 1). Furthermore, the ability of the organism to utilize different carbon sources was also studied and the results are given in Table 2. Microscopic examination of the sporophores revealed long wavy chains which lacked spirals (Plate 1). The electron-micrograph demonstrated cylindrical spores with rough irregular surfaces (Plate 2).

Streptomyces AS-Y-400 differs from *S. alboviridis*, *S. griseoviridis*, *S. prusinus*,

Plate 1. Sporophores of *Streptomyces* AS-Y-400 (×1,000×1/1.5)

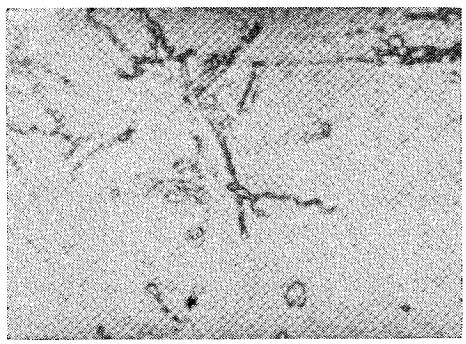


Plate 2. Electron micrograph of spores of *Streptomyces* AS-Y-400 (×16,000×1/1.5)

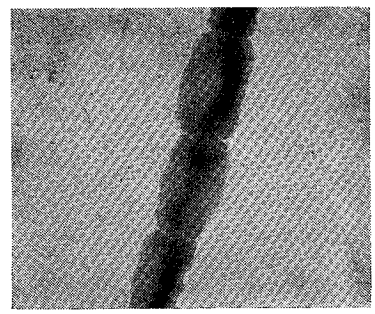


Table 1. Culture characteristics of *Streptomyces* AS-Y-400

Medium	Characteristics
Starch-nitrate	G. good A. grey velvety Sub. pale green S. dirty green
Glucose-nitrate	G. good A. pale grey velvety Sub. pale yellowish brown S. brown
Glucose-asparagin	G. good A. pale grey velvety Sub. pale green S. green
Glycerol-asparagin	G. good A. pale grey velvety Sub. pale orange yellow S. greenish brown
Potato plug	G. very good A. grey velvety Sub. pale brown S. blackish green
Nutrient-agar	G. weak A. none Sub. pale orange yellow S. none
H_2S formation	G. weak A. none Sub. creamy S. none
Milk	G. moderate slow coagulation without peptonization
Nitrate reduction	rapid reduction after 3 days
Gelatin liquefaction	rapid liquefaction
Cellulose decomposition	G. good decompose cellulose with the rise of grey aerial mycelium
Melanine formation	S. dark brown (negative)

Where : G. =growth. A. =aerial mycelium.
Sub. =substrate mycelium. S. =soluble pigments.

Table 2. Utilization of carbon sources

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

+++ good growth, ++ moderate growth, + feeble growth

S. viridis and *S. hirustus* which were classified by WAKSMAN[1] as members of the Viridis series. They are all similar in having light grey aerial hyphae but the last three lack antagonistic properties which make them distinctly different from AS-Y-400. The sporophores of theses organisms are either straight or spiral while those of AS-Y-400 are wavy. *Streptomyces* AS-Y-400 differs from *S. alboviridis* and *S. griseoviridis* in lacking spiral sporophores. Further distinction between AS-Y-400 and *S. alboviridis* could be made by the greyish olive green pigment secreted in starch-nitrate medium by AS-Y-400. The aerial mycelia on glycerol-asparagin medium are pale grey velvety in culture of AS-Y-400 and pink to grey green in case of *S. griseoviridis*.

Production, Isolation and Purification of Hodydamycin

Streptomyces AS-Y-400 grew well at 28°C in shaken cultures (200 r.p.m.). The medium used for the production of hodydamycin contained the following ingredients (g/100 ml) : 1.0, glucose ; 0.2, $NaNO_3$; 0.2, K_2HPO_4 ; 0.05, $MgSO_4 \cdot 7H_2O$ and 0.05, KCl.

The pH of the medium was adjusted to 7.0 before sterilization. The antibiotic contents reach a maximum (150 mg/liter) after 4~6 days.

The broth was filtered and extracted with a mixture of ethyl acetate and chloroform (1 : 1) at pH 7.5. The extract was evaporated in vacuum till dryness. The residual powder had a pale olive green colour and a detectable antimicrobial activity. This crude product was dissolved in ethylacetate which was then shaken vigorously with carbonate buffer of pH 9.5. The organic phase was separated from the buffer solution which was darkly stained with olive green pigment. The ethylacetate solution was washed with distilled water and concentrated in vacuum. The antibiotic was precipitated by addition of petroleum ether (40~60°C). Although the purity of the product was markedly improved, paper chromatographic analysis showed the presence of other inactive impurities.

Fig. 1. Ultraviolet absorption spectrum of hodydamycin.

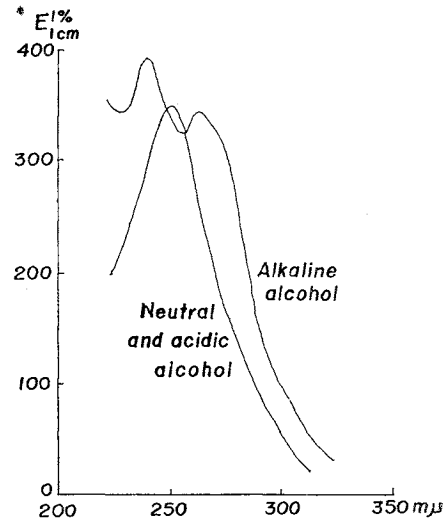
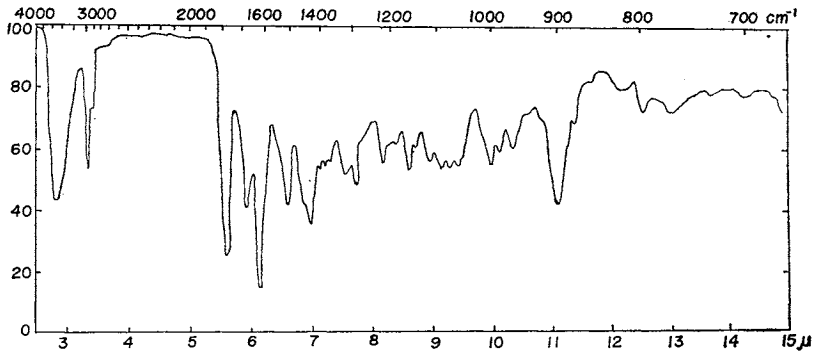


Fig. 2. Infrared absorption spectrum of hodydamycin (KBr).



Final purification was achieved by dissolving the crude antibiotic in absolute ethanol and passing the solution through a short column of activated charcoal. The eluate was concentrated in vacuum and then diluted with distilled water until turbidity appeared. The mixture was cooled overnight and the pure antibiotic precipitated as colourless needles.

Physical and Chemical Properties

Physical properties

The pure antibiotic melts at 145°C. It is readily soluble in common organic solvents, but insoluble in petroleum ether and water. The ultraviolet absorption spectrum in neutral and acidic ethanol had a maximum at 249 mμ ($E^{1\%}_{1cm}$ 358) while in alkaline alcohol it showed maxima at 237 and 263 mμ ($E^{1\%}_{1cm}$ 400 and 350 respectively)

(Fig. 1). The infrared absorption spectrum of the antibiotic in KBr pellet exhibited characteristic absorption bands at 3500, 2930, 1780, 1680, 1630, 1520 and 1430 cm^{-1} (Fig. 2). Optical rotation of the ethanolic solution of hodydamycin was $[\alpha]_D^{25}$ +40°. The Rf values on descending paper chromatograms when using different developing solvents are expressed in Fig. 3. Location of the zones occupied by the antibiotic was determined bioautographically using *B. subtilis* as the test organisms. One definite inhibition zone was always observed.

Chemical properties

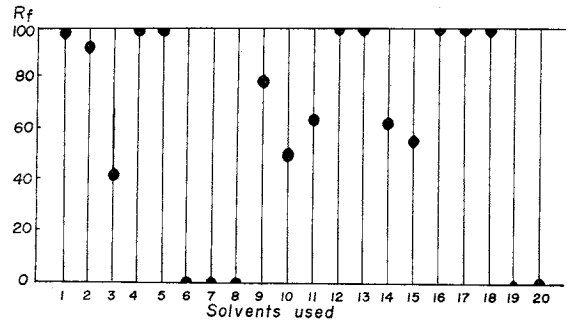
Elemental analysis yielded the following data:

Calculated for $C_{40}H_{50}N_3O_{14}Cl$:
C 57.74, H 6.01, N 5.05, O 26.93, Cl 4.27%

Found:
C 57.76, H 6.06, N 4.96, O 26.91, Cl 4.31%

Behaviour of the antibiotic towards different chemical tests (Table 3) confirms its peptide nature, the presence of strongly absorbing structures for bromine and iodine and the absence of sugar moieties. On hydrolysing the pure antibiotic with 6 N HCl at 105°C for 24 hours the following aminoacids could be identified by paper chromatography: glycine, lysine, aspartic acid, alanine and 3 hydroxypoline (fast moving). No other degradation products could be detected by the analytical technique applied.

Fig. 3. Migration of hodydamycin on paper chromatograms using different developing solvents.



No.	Solvent used
1	Ethyl acetate
2	Ethyl acetate : water (1 : 1)
3	Ethyl acetate : petroleum ether : water (5 : 2.5 : 5)
4	Ethyl acetate : ammonia (1 : 1)
5	Ethyl acetate : acetic acid (1 : 1)
6	NH_4Cl 3%
7	Distilled water
8	Na_2HPO_4 0.5%
9	*n*-Butanol
10	*n*-Butanol : petroleum ether : water (5 : 2.5 : 5)
11	*n*-Butanol : ethyl alcohol : water (5 : 2.5 : 5)
12	*n*-Butanol : acetic acid : water (4 : 1 : 5)
13	Chloroform
14	Chloroform : ethyl alcohol : water (5 : 2.5 : 5)
15	Chloroform : petroleum ether : water (5 : 2.5 : 5)
16	Chloroform : water (1 : 1)
17	Chloroform : ammonia (1 : 1)
18	Chloroform : acetic acid (1 : 1)
19	Petroleum ether
20	Na_2CO_3 0.5%

Table 3. Behavior of hodydamycin towards different chemical tests

Chemical test	Result
Ninhydrin	positive
$FeCl_3$	negative
Alkaline $KMnO_4$	reduction on cooled
Acidic $KMnO_4$	〃
Biuret	positive
MOLISCH	negative
Nitration	negative
Reduction to FEHLING	negative
Potassium thiocyanate	negative
Potassium ferrocyanide	negative
Dilute I_2 solution	decolorization on cooled
Dilute Br_2 solution	〃
SAKAGUCHI	negative
TOLLENS	negative
MILLON	negative

Biological Properties

The minimum inhibitory concentrations (MIC) of hodydamycin for a variety of microorganisms are given in Table 4. Determination of the MIC was carried out with the following medium (g/100 ml): 0.15, yeast extract; 0.15, beef extract; 0.5, peptone;

Table 4. Antimicrobial spectrum of hodydamycin using serial agar dilution method

Test organism	M.I.C. (mcg/ml)	Test organism	M.I.C. (mcg/ml)
Bacillus subtilis 24G	0.75	*Salmonella typhosa*	>100
Bacillus subtilis 19T	0.37	*Salmonella paratyphi* A-2: a	>100
Bacillus subtilis D_{161} (chlortetracycline R.)	1.5	*Salmonella enteritidis* Univ. Ill.	>100
Bacillus subtilis AA	0.75	*Escherichia coli* 0127: B_8; H-VCNCTC 9609	>100
Bacillus subtilis ICC	0.37		
Bacillus subtilis D_{161} (novobiocin R.)	1.5	*Escherichia coli* 0127: B_8 MJ 50	>100
Bacillus subtilis D_{161} (chloramphenicol R.)	1.5	*Escherichia coli* N 27405	>100
Bacillus subtilis D_{161} (streptomycin R.)	0.25	*Escherichia coli* D_{165}	>100
Bacillus subtilis D_{161} (staphylomycin R.)	1.5	*Escherichia coli* NRRL B-210	>100
Bacillus subtilis NRRL-B-543	1.5	*Klebsiella pneumoniae* NRRL B-36	6.25
Bacillus subtilis D_{161} (spiramycin R.)	3.12	*Klebsiella pneumoniae* O-1-K3NCTC 5056	12.5
Bacillus mycoides (U.S.S.R.)	12.50		
Bacillus cereus D_{166} (oxytetracycline R.)	25.0	*Klebsiella pneumoniae* NRRL B-117	>100
Bacillus cereus D_{166} (kanamycin R.)	50.0	*Klebsiella pneumoniae* 1231-67-CDC	50
Bacillus cereus NRRL B-596	50.0	*Shigella boydii* 22854-61-CDC	50
Bacillus diphtheroid	>100	*Shigella equirulis* H-33	25.0
Staphylococcus aureus D_6 (paromycin R.)	6.2	*Proteus vulgaris* Pr. 1 CDC	6.2
Staphylococcus aureus A_{55}	3.2	*Proteus mirabilis* Su I_2	6.2
Staphylococcus aureus D_{66} (PKAM R.)	1.5	*Proteus mirabilis* H-3	50.0
Staphylococcus aureus D_6 (oleandomycin R.)	1.5	*Proteus rettgeri* SuI_9	>100
Staphylococcus aureus D_6 (streptomycin R.)	3.2	*Pseudomonas aeruginosa* D_{129}	>100
Staphylococcus aureus NRRL B-313	6.2	*Pseudomonas aeruginosa* SuI 14	>100
Staphylococcus rosea	12.5	*Pseudomonas aeruginosa* ATCC 14502	>100
Staphylococcus aureus FDA 290P	25.0	*Pseudomonas aeruginosa* 9027	>100
Staphylococcus aureus I-42/3	25	*Haemophilus influenzae* A-733	1.5
Corynebacterium hoffmanii	0.31	*Enterobacter aerogenes* 659-66-CDC	50.0
Corynebacterium michiganense NRRL B-33	3.12	*Saccharomyces cerevisiae* NRRL Y-567	>100
Corynebacterium minutissimum UP 54	>100	*Candida albicans* NRRL Y-477	>100
Salmonella paratyphi C-6, 7: C	>100	*Aspergillus niger*	>100
Salmonella paratyphi B-4, 6: b	>100		
Salmonella typhosa NRRL B-573	>100		

Table 5. Effect of inoculum size on the MIC

Test organism	Inoculum level	MIC (mcg/ml)
Staphylococcus aureus (Smith)	10^5	25
	10^3	6.25
	10^1	3.12
Bacillus subtilis (AA)	10^5	3.12
	10^3	0.75
	10^1	0.35
Corynebacterium hoffmanii	10^5	6.25
	10^3	0.75
	10^1	0.36

0.1, glucose; 0.13, K_2HPO_4 and 0.35, NaCl and the pH was adjusted to 7 before sterilization.

The results shown in Table 4 demonstrate that hodydamycin is mainly active against Gram-positive bacteria while very limited activities were shown against Gram-negative organisms. The effect of inoculum size was also examined and the results are given in Table 5. The antibiotic effect was markedly dependent on the inoculum size.

Finally the *in vivo* tests showed that the antibiotic failed to exert any suppressing influence against *S. aureus* infections except when administrated via the oral route at concentrations of 180 mg/kg body weight (CD_{50}=180). This exceptionally high CD_{50} suggests a possible high serum-binding property for the antibiotic. Such a possibility was confirmed by studying different dilutions of the antibiotic solution with a 50 % diluted horse serum. The antibiotic failed to exert any antibacterial effect against

S. aureus FDA 209P even at concentrations up to 100 mcg/ml.

Hodydamycin possesses a low toxicity. The LD_{50} for Swiss mice via the intraperitoneal route amounted to 1.0 g/kg body weight.

Discussion

Morphological and physiological characters of strain AS-Y-400 seem to differentiate from *S. alboviridis*, *S. griseoviridis*, *S. viridis S. prasinus* and *S. hirustus* of the viridis series.

Hodydamycin is a peptide which is freely soluble in most organic solvents but hardly soluble in water and petroleum ether. The acid hydrolysate of the antibiotic yielded five aminoacids, namely glycine, lysine, aspartic acid, alanine and 3 hydroxy proline.

Hodydamycin differs from other polypetide antibiotics which are soluble in organic solvents[2]. Etamicin[3], ostreogrycin A (E-129A)[4] pyridomycin[5] and ilamycin[6] are distinctly different from hodydamycin with regard to biological activity, acid hydrolytic products and physical properties. Telomycin[7] showed some resemblance to hodydamycin; both are mainly active against Gram-positive bacteria and the acid hydrolytic products of hodydamycin (5 aminoacids) also found among ten aminoacids which constitute the hydrolytic products of telomycin. Nevertheless, telomycin showed absorption peak at 340 mμ ($E^{1\%}_{1cm}$ 127) while that of hodydamycin lay at 249 mμ in neutral and acidic ethanol.

Despite the failure to detect among the hydrolytic products of hodydamycin any products other than the aminoacids, the existence of unsaturated moieties is strongly indicated by infrared spectrum as well as by the bromine and iodine absorption. In view of this the comparatively low proportion of nitrogen compared to that anticipated from a pure polypeptide would be explained, actinogen[8], antibiotic 1415[9] and amidomycin[10] contain several aminoacids in their hydrolytic products even though the elemental analysis of these products gave comparatively low proportions of nitrogen.

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