A-16686, A NEW ANTIBIOTIC FROM *ACTINOPLANES*

I. FERMENTATION, ISOLATION AND PRELIMINARY PHYSICO-CHEMICAL CHARACTERISTICS†

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Actinoplanes sp. ATCC 33076 is a new strain that was found to produce an antibiotic, designated A-16686, which is a complex of three closely-related polypeptides containing chlorinated phenyl moieties and D-mannose. Both the complex and the single fractions possess a good activity against Gram-positive bacteria. A-16686 specifically inhibits the synthesis of the bacterial cell wall.

In the course of our screening for antibiotics from *Actinoplanes* we isolated a strain which produces an antibiotic designated as A-16686 from a soil sample collected at Vaghalbod Village, Indore, India.

Taxonomic studies showed that the strain is a new species of *Actinoplanes* which has been deposited in the American Type Culture Collection as ATCC 33076. A-16686 is active against Gram-positive bacteria.

The taxonomy of the producing organism, fermentation and isolation of the antibiotic complex are reported in the present paper. The single factors were separated from each other by high performance liquid chromatography. Some physicochemical and biological properties are also described.

Taxonomy of the Producing Organism

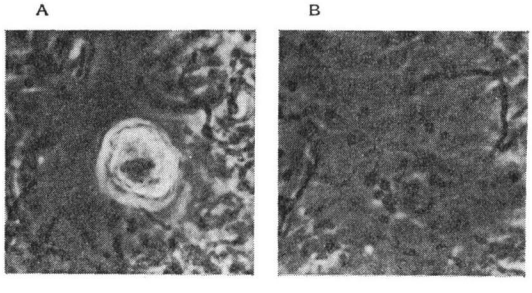
Morphological Properties

The morphology of the culture in agar media is typical of *Actinoplanes*. The strain grows well on different media with an orange color of the substrate mycelium. It does not produce pigments. Aerial mycelium is always absent.

Under microscopic examination the vegetative mycelium reveals branched hyphae with a diameter of about 1.0 μm. The sporangia form scantily on potato agar only and are globose with a very irregular surface and a diameter ranging from 5.0 to 9.0 μm. Sporangial release is observed after rupture of the wall of the sporangium (Fig. 1). The subspherical spores are motile (1.0~1.5 μm diameter). Analysis of the cell wall components reveals *meso*-diaminopimelic acid and a sugar pattern of type D[1].

Fig. 1.

A: Sporangium obtained on potato agar.
B: Spores, immediately after release from sporangium.
Magnification ×1,200.



† Some of these data were presented at the 13th International Congress of Chemotherapy, Vienna, August 28, 1983.

Cultural Characteristics

The cultural properties of *Actinoplanes* sp. ATCC 33076 on different media suggested by SHIRLING and GOTTLIEB[2] and by WAKSMAN[3] are shown in Table 1.

The characteristics of the culture were determined after 6~14 days of incubation at 30°C.

Table 1. Cultural characteristics of strain ATCC 33076.

Culture media*	Cultural characteristics**
Medium No. 2 (yeast extract - malt agar)	Abundant growth with wrinkled surface, light brown 12 H 12
Medium No. 3 (oatmeal agar)	Scant growth, thin, light orange 9 B 6
Medium No. 4 (inorganic salts - starch agar)	Moderate growth, crusty surface, orange 11 L 12
Medium No. 5 (glycerol - asparagine agar)	Scant growth, hyaline
Medium No. 6 (peptone - yeast extract - iron agar)	Scant growth, hyaline to light brown
Medium No. 7 (tyrosine agar)	Scant growth, smooth surface, brown 6 D 11
Oatmeal agar (according to WAKSMAN)	Abundant growth with wrinkled surface, orange to brown 12 C 10
HICKEY and TRESNER's agar.	Abundant growth, crusty surface, orange 11 G 8
Czapeck glucose agar	Moderate growth, crusty surface, orange 11 G 8
Glucose asparagine agar	Scant growth, crusty surface, light orange 11 F 6
Nutrient agar	Moderate growth, smooth surface, orange 11 G 8
Potato agar	Abundant growth, wrinkled surface, amber-brown 12 E 10
Bennett agar	Abundant growth, wrinkled surface, orange 11 G 8
Calcium malate agar	Moderate growth with smooth surface, light orange 10 C 6
Skim milk agar	Abundant growth with wrinkled surface, orange 9 L 2
Czapeck agar	Moderate growth, crusty surface, orange 10 D 7
Egg agar	Moderate growth, smooth surface, hyaline to light orange
Peptone glucose agar	Abundant growth, wrinkled surface, orange 11 G 11
Agar	Very scant growth, smooth surface, hyaline
Loeffler serum	Very scant growth, smooth surface, orange
Potato	Scant growth, crusty, light brown
Gelatin	Scant growth, light orange
Cellulose	Very scant growth, thin, hyaline

* Numbers used for some culture media refer to those given by SHIRLING and GOTTLIEB[2].

** Letters and numbers refer to the color determined according to MAERZ and PAUL[4].

Table 2. Utilization of carbon sources by strain ATCC 33076.

Carbon source	Utilization
Inositol	−
Fructose	+
Rhamnose	+
Mannitol	−
Xylose	+
Raffinose	−
Arabinose	+
Cellulose	−
Sucrose	+
Glucose	+
Mannose	+
Lactose	−
Salicin	+

+, Positive utilization; −, no growth.

Table 3. Physiological characteristics of strain ATCC 33076.

Test		Results
Hydrolysis of starch		Positive
H_2S formation		Positive
Tyrosinase reaction		Negative
Casein hydrolysis		Positive
Solubilization of calcium malate		Negative
Liquefaction of gelatin		Positive
Litmus milk	Coagulation	Positive
	Peptonization	Negative
Cellulose decomposition		Negative

Carbon Utilization

The utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[5] (Table 2).

Sensitivity to Temperature

A temperature range of about 22° to 42°C was found to be most suitable for the development of colonies, the optimum temperature being between 28°C and 37°C.

Physiological Characteristics

The physiological characteristics are reported in Table 3.

Fermentation Studies

A typical time-course fermentation of strain A-16686/2, a natural variant of the wild type ATCC 33076, in a 300-liter fermentor containing 200 liters of medium FS/9 (Table 4) is shown in Fig. 2. The operative conditions are described below. The antibiotic activity was assayed by the agar diffusion method using *Staphylococcus aureus* ATCC 6538 as test organism both in the filtered broth and in the methanol extracts from the collected mycelium and referred to the harvested broth samples.

Fig. 2. Time-course of the fermentation with a 300-liter fermentor of A-16686/2 strain.

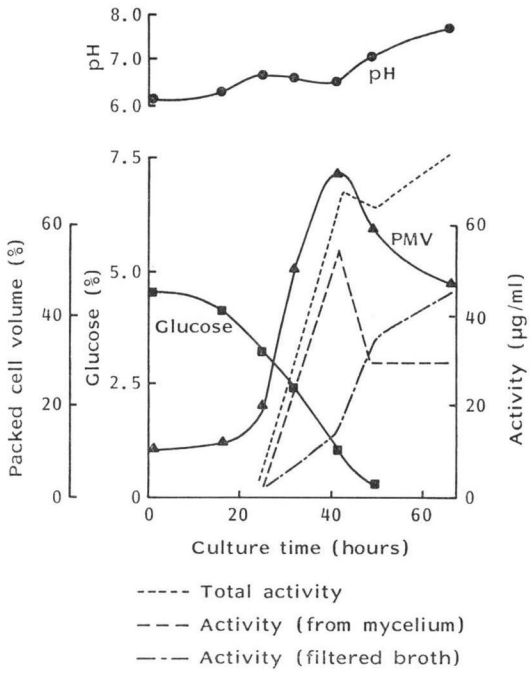


Table 4. Seed medium and fermentation medium.

Seed medium (VM), %		Production medium (FS/9), %	
Meat extract	0.3	Soybean meal	3
Yeast extract	0.5	Glucose	4
Tryptone	0.5	$CaCO_3$	1
Soluble starch	2.4		
Glucose	0.1		
$CaCO_3$	0.4		

Production and Isolation

The titers of the antibiotic in the powders and of the final product were measured both by microbiological assay against *S. aureus* ATCC 6538 and by HPLC determination (Table 5, Fig. 3).

TLC of the eluted fractions was run as described in Table 6.

Although in some fermentations a variable amount of antibiotic was recovered by extraction of the filtered broth after acidification, the procedure described below refers to the treatment of the mycelial mass which contains most of the activity.

One hundred milliliters of the 96 hour-old culture in a 500-ml flask was inoculated into a 10-liter jar fermentor containing 4 liters of seed VM medium (Table 4) and was cultivated at 28°C for 48 hours using an agitator rate of 900 rpm and an aeration rate of 1 vol/vol/minute. Three liters of the seed preculture were transferred into a 50-liter stainless steel fermentor containing 30 liters of VM medium

and cultivated at 28°C for 24 hours (agitation rate 500 rpm, aeration rate 1 vol/vol/minute). Twenty liters of the above culture was used to inoculate a 300-liter stainless steel fermentor containing 200 liters of FS/9 production medium (Table 4). The fermentation was run at 28°C for 24 hours (agitation rate 250 rpm, aeration rate 1 vol/vol/minute).

The fermentation broth was cooled at 10°C, brought to pH 3.5 with conc HCl and filtered with a rotating filter precoated with CLARCEL FLO-MA filter-aid. The mycelial cake was then extracted with 60 liters of methanol under stirring for one hour, filtered and re-extracted with 50 liters of 90% aqueous methanol. The exhausted mycelium was discarded after filtration and the combined extracts were concentrated under reduced pressure at 40°C to a volume of 10 liters. The pH was adjusted to 3.5 with 18% HCl and the solution was washed with 10 liters of ethyl acetate, then extracted with 10 liters of butanol. After concentration of the butanol extract under reduced pressure to a residual volume of 1 liter, the solution was cooled at 5°C overnight. The precipitate was filtered off, washed with ethyl ether and dried obtaining 40.4 g of crude antibiotic having an activity titer of 24.7%. The raw material was treated with 270 ml of a chloroform - ethanol - water mixture (4: 7: 3). The oily product that formed was separated from the solution by decantation, treated again with 60 ml of the same mixture, and triturated with 135 ml of water. A tractable precipitate was formed that was collected by centrifuging, then it was suspended in water (160 ml) and dissolved by adding methanol (370 ml).

The solution was filtered to remove a small amount of insoluble matter, a few drops of 1 N HCl was added and the solution was stripped under reduced pressure at a temperature less than 35°C with the addition of butanol.

Addition of ethyl ether to the butanol concentrate gave a precipitate that was filtered off, washed with a small amount of ethyl ether and dried under reduced pressure at room temperature (12.6 g; activity titer 54.3%). A solution of the product in 800 ml of 50% aqueous acetonitrile was adsorbed on a column containing 3.5 kg of silica gel (Merck Co., 0.06~0.2 mm) prepacked with the same solvent. The column was eluted using the same acetonitrile - water mixture, then with acetonitrile - 0.01 N HCl, 1: 1.

The active fractions were combined and the solvents were evaporated to a small volume at reduced pressure after addition of butanol.

The addition of ethyl ether afforded a precipitate which was filtered off, dissolved in 320 ml of 30% aqueous methanol and applied to a column containing 1.9 kg of Sephadex LH-20 (Pharmacia) prepared in the same solvent. The column was developed with the same solvent and the bioactive fractions were combined and concentrated as described above. After adding a few drops of 1 N HCl to the butanol concentrate the antibiotic was precipitated with ethyl ether, filtered and dried under reduced pressure at 35°C, obtaining 4.25 g of the hydrochloride.

The free base was prepared by adding 2 ml of 1,2-epoxybutane to a solution of 50 mg of A-16686 in 11 ml of 28% aqueous ethanol. By allowing the mixture to stand at room temperature for one day a precipitate formed that was centrifuged, washed with ethanol and dried under reduced pressure over P_2O_5 (16 mg).

Separation of Factors A1, A2, A3

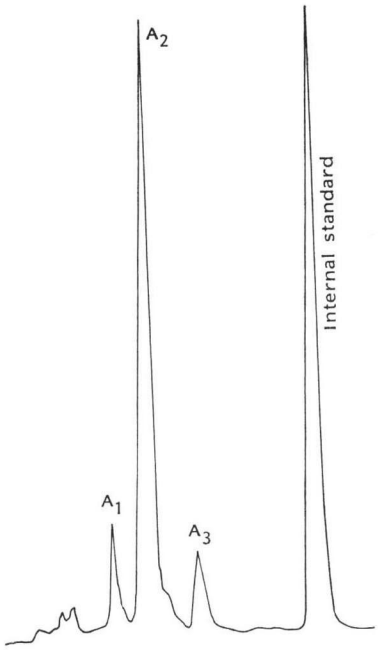
Both reverse-phase TLC (Table 6) and HPLC (Fig. 3) showed that the A-16686 obtained as described above contains three factors designated A1, A2 and A3, in a 6÷12/86÷74/8÷14% ratio, respectively (percentages of HPLC peak areas).

The components were separated by semi-preparative HPLC under the conditions indicated in Table 5. A solution of 169 mg of the complex in 6.5 ml of 0.01 N HCl was diluted with 10.4 ml of water. Repeated injections of 1 or 2 ml were made collecting the fractions containing the single factors that were further checked by means of analytical HPLC and then combined. The evaporation in the presence of a small amount of butanol gave a residue that was dissolved in distilled water and lyophilized. This operation was repeated until no more $HCOONH_4$ was present, as determined by Nessler test, yielding pure factors A1 (10 mg), A2 (95 mg) and A3 (12 mg). By lyophilizing a 10% hydrochloric solution of A-16686 factor A2, the corresponding hydrochloride was obtained as an amorphous powder melting at 250°C (dec).

Fig. 3. HPLC profile of A-16686 (the operative conditions are reported in Table 5).

The compound was dissolved (1 mg/ml) in a mixture CH_3CN - H_2O, 1: 1.

Internal standard, 1-nitronaphthalene.



Physico-chemical Properties

The A-16686 complex and single factors A1, A2 and A3 are white powders, slightly hygroscopic and very soluble in water as hydrochlorides, soluble in DMF and lower alcohols, insoluble in ethyl ether, petroleum ether and benzene. A-16686 gave a positive ninhydrin reaction (in the presence of sodium acetate) and positive Molish, biuret, anthrone, $FeCl_3$-$K_3Fe(CN)_6$ (green color) reactions.

Some physico-chemical properties are reported in Table 7. The elemental analysis showed the presence of C, H, N and chlorine. An equivalent weight of about 1,070 was obtained from potentiometric titration of a sample of the complex in Methyl Cellosolve - AcOH solution with 0.01 N $HClO_4$ in the presence of mercuric acetate.

Table 5. HPLC of A-16686.

	Analysis	Separation
Apparatus	Varian 5000 pump, Rheodyne Mod. 7125 (loop 10 μl) injector	Waters M45 pump, Waters U6K (loop 2 ml) injector
Stationary phase	μBondapak C_{18} (Waters)	μBondapak C_{18} (Waters)
Length of the column	300 mm	300 mm
Internal diameter of the column	3.9 mm	7.8 mm
Mobile phase	$HCOONH_4$ (0.025 M) - CH_3CN, 60: 40	$HCOONH_4$ (0.025 M) - CH_3CN, 65: 35
Column temperature	Ambient	Ambient
Column pressure	141 kg/cm^2	141 kg/cm^2
Flow rate	2 ml/minute	4 ml/minute
Detector	Perkin-Elmer LC 15 (254 nm)	Perkin-Elmer LC 55 (295 nm)
Integrator	SP 4000 Data System (Spectra Physics)	

Table 6. Paper and thin-layer chromatographic behavior of A-16686 complex and of factors A1, A2, A3.

	Elution systems*	Rf values			
		A-16686 complex	A1	A2	A3
1)	1-BuOH satd with Sörensen buffer, pH 6.0	0.0	0.0	0.0	0.0
2)	1-BuOH satd with H_2O containing 2% of *p*-toluenesulfonic acid	0.0	0.0	0.0	0.0
3)	1-BuOH satd with H_2O containing 2% of NH_4OH	0.0	0.0	0.0	0.0
4)	Sörensen buffer, pH 6.0, satd with 1-BuOH	0.05	0.05	0.05	0.05
5)	1-BuOH - AcOH - H_2O, 2: 1: 1	0.48	0.44	0.44	0.44
6)	1-BuOH - AcOH - H_2O, 4: 2: 5	0.54	0.52	0.52	0.52
7)	1-PrOH - 1-BuOH - 1 N NH_4OH, 2: 3: 4, upper layer	0.12	0.12	0.12	0.12
8)	1-BuOH - AcOH - H_2O, 4: 1: 1	0.17	0.15	0.15	0.15
9)	1-BuOH - AcOH - H_2O, 4: 2: 5	0.80	0.80	0.80	0.80
10)	Aqueous 2.5% $HCOONH_4$ - acetonitrile, 65: 35**	0.40, 0.36, 0.32	0.40	0.36	0.32

* 1~5: Descending chromatography (40 cm) on Whatman No. 1 paper visualized by microbiological assay with *Bacillus subtilis* ATCC 6633.
6~8: TLC (140 mm) on silica gel 60 F_{254} plates (Merck). Visualization by exposure to iodine vapor or to UV light (254 nm).
9,10: TLC (140 mm) on silanized silica gel 60 F_{254} (Merck). Visualized as above.

** Internal reference caffeine (Rf 0.60).

Table 7. Physico-chemical properties of A-16686 complex and of factors A1, A2, A3.

	A-16686 complex	A1	A2	A3
Mp (°C)[a]	210~230	210~220	210~220	210~220
$[\alpha]_D^{20}$	+78.3° (*c* 1.04, H_2O)	+57±4° (*c* 0.51, H_2O)	+73±4° (*c* 0.49, H_2O)	+50±4° (*c* 0.48, 0.01 N HCl)
Anal[b] (%) C	52.54	50.31	53.40	48.41
H	6.55	6.00	6.12	5.84
N	9.98	9.92	10.74	9.09
Cl (total)	3.70	1.61	1.37	1.68
Cl^-	3.30	0.90	Absent	1.16
Weight loss, %	2.00	9.00	6.40	9.60
Residue, %	0.70	6.70	2.18	9.07
HPLC retention time (minutes)[c]	—	3.84	4.99	6.89

[a] Determined by differential scanning calorimetry.
[b] C, H, N were determined on samples previously dried at 140°C in inert atmosphere. The weight loss was measured by thermogravimetric analysis. The inorganic residue was determined after heating the samples at 900°C in oxygen atmosphere.
[c] See Fig. 3. Internal standard, 1-nitronaphthalene (retention time, 11.14 minutes).

This value, after correction for solvents and inorganic residue, corresponded to an approximate molecular weight of 2,400. The amino acid analysis of the acid hydrolysate (6 N H_2SO_4, 110°C, 6 hours) of A-16686 complex revealed the presence (μmol/mg) of alanine (0.32), leucine (0.31), glycine (0.33), aspartic acid (0.22), phenylalanine (0.25), ornithine (0.58), threonine (0.57) and four additional unidentified acid or neutral amino acids.

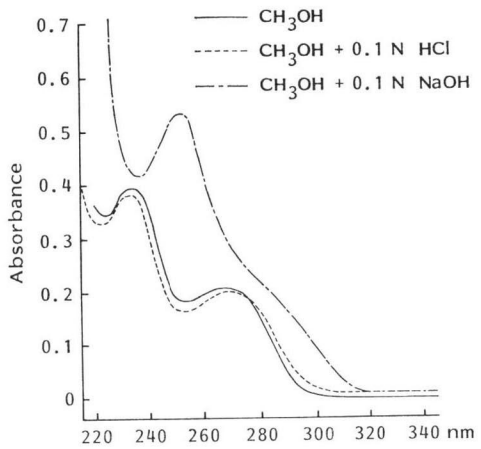
GC and GC-MS analysis of the hydrolysate after derivatization of the amino acids as *N*-trifluoroacetylmethyl esters confirmed the residues listed above and allowed the assignment of the structures of *p*-hydroxyphenylglycine and hydroxychlorophenylglycine to two of the unidentified amino acids.

Furthermore, the acid hydrolysates of the complex and of the single factors were compared, after

Table 8. Comparison of some of the characterized amino acids of A16686 complex and factors A1, A2, A3. The percentages were calculated on the areas of the peaks obtained by GC of the acid hydrolysis mixtures derivatized as *N*-trifluoroacetyl-methyl esters.

Amino acids	Complex (%)	A1 (%)	A2 (%)	A3 (%)
Alanine	3.5	4.7	4.4	4.1
Leucine	8.5	9.7	9.2	8.6
Glycine	2.6	2.9	3.1	3.0
Aspartic acid	2.8	3.3	6.2	5.4
Phenylalanine	9.7	11.1	11.7	9.0
Ornithine	12.0	13.3	15.2	13.1
Hydroxychloro-phenylglycine	14.5	14.2	11.2	17.5
p-Hydroxyphenyl-glycine	46.3	40.6	39.2	39.2

Fig. 4. UV spectrum of A-16686 factor A2.



derivatization, as shown in Table 8. The relative amounts of some amino acids seem to vary among the three factors.

Sugar determination was made on the complex and the single factors after hydrolysis (2 N H_2SO_4, 100°C, 2 hours) and neutralization with $Ba(OH)_2$. The analysis with different techniques (paper and TLC chromatography; GC-MS of the hydrolysate after silanization by heating in a mixture of chloro-trimethylsilane - bis-silylacetamide, 1: 3 in anhydrous pyridine for 1 hour at 80°C) showed the presence of D-mannose.

A quantitative analysis was carried out on the acid hydrolysate (2 N H_2SO_4, 120°C, 1 hour) of the complex by high performance thin-layer chromatography after neutralization. From 44.66 mg of a sample of the complex (titer 80.5%) 4.85 mg of D-mannose was determined which, assuming a MW of 2,400, corresponded to 1.8 mol of D-mannose/mol of A-16686.

The UV spectrum of factor A2 (Fig. 4) showed maxima at 234 ($E^{1\%}_{1cm}$ 206) and 268 nm (114) in MeOH and in MeOH plus 0.1 N HCl, shifted to 251 ($E^{1\%}_{1cm}$ 275) and 290 nm (shoulder), respectively, in MeOH plus 0.1 N NaOH.

Fig. 5. IR spectrum (Nujol) of A-16686 factor A2.

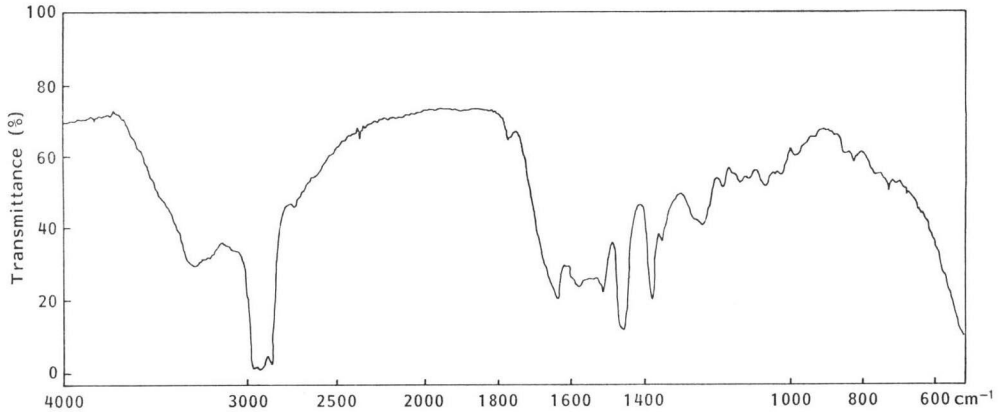


Fig. 6. ^{1}H NMR spectrum at 270 MHz of A-16686 factor A2 in DMF-d_7 - D_2O solution.

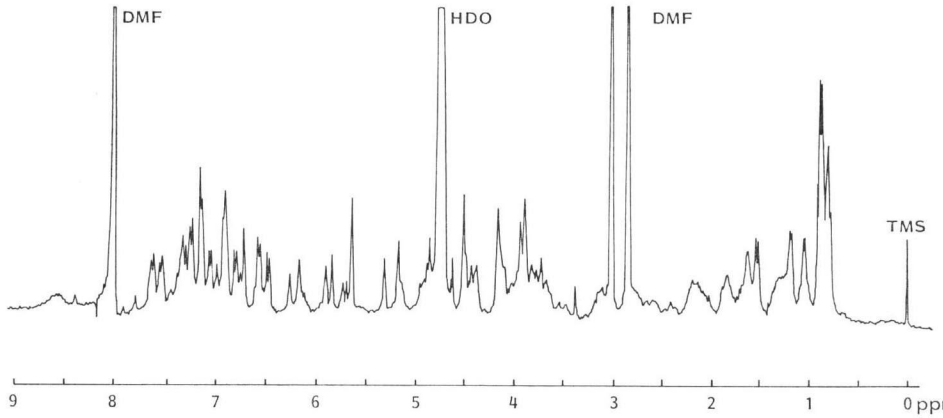


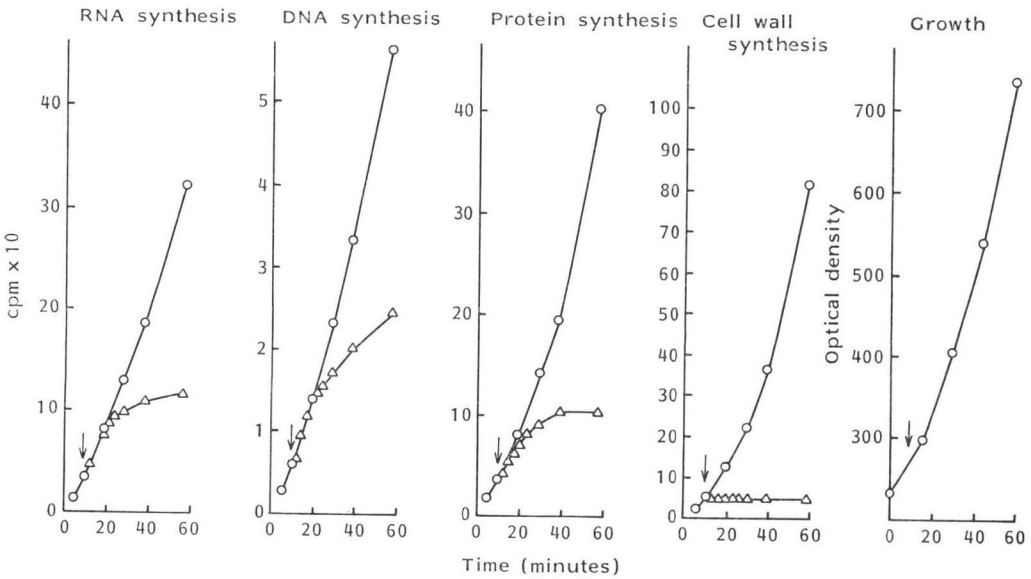
Fig. 7. Effect of A-16686 on macromolecular synthesis in *B. subtilis*.

Radioactively labelled precursors were added at zero time to cultures of *B. subtilis* Thy$^-$ growing logarithmically in a defined medium. Antibiotic (0.5 μg/ml) was added 10 minutes later and the incubation continued for one generation time (~48 minutes). Incorporation of radioactivity into the cold 5% trichloroacetic acid insoluble fraction of cells was determined at suitable intervals before and after addition of A-16686 and also in a control culture containing no antibiotic. Growth was determined by measuring the absorbance at 575 nm in 1 cm cuvettes. Acid insoluble material was collected on membrane filters and the radioactivity estimated by liquid scintillation counting.

The radioactive precursors used were as follows: [*U*-^{14}C]Phenylalanine for protein synthesis (0.66 μCi/ml, 5 μg/ml); [5-^{3}H]uracil for RNA synthesis (0.1 μCi/ml, 5 μg/ml); [2-^{14}C]thymidine for DNA synthesis (0.2 μCi/ml, 10 μg/ml); *N*-[1-^{3}H]acetylglucosamine for cell wall synthesis (0.131 μCi/ml, 5 μg/ml).

○ Control

△ A-16686 (0.5 μg/ml)



The IR spectrum of factor A2 (Fig. 5) in Nujol showed absorption bands at 1630 ($\nu_{C=O}$, amide I), 1510 (δ_{NH}, amide II), 1065~980 (ν_{C-O}, sugars), 840 and 820 cm^{-1} (γ_{CH} aromatic).

The characteristics reported above are practically the same for the complex and the A1 and A3 factors.

The ^{1}H NMR spectrum* of factor A2 (Fig. 6) showed approximately 125 non mobile protons: 18 H in the region δ 0.8~1 ppm, attributable to six methyl groups of three leucine units; 32 H in the region δ 1~3 ppm on carbons bonded to carbon atoms, among them three methyl groups [three doublets centered at δ 1.07 ppm (J=5 Hz, 3H), δ 1.20 ppm (J=6 Hz, 3H) and δ 1.53 ppm (J=7 Hz, 3H), respectively] attributable to two alanine and one threonine units; 40 H on carbons bonded to oxygen or nitrogen and of olefinic type in the region δ 3~6.4 ppm, among them the signals at δ 5.12 ppm and at δ 5.28 ppm tentatively attributable to the anomeric protons of two D-mannose units; 35 H of aromatic type in the region 6.4~8.5 ppm, among them four doublets [centered at δ 6.42 (J=8.5 Hz, 2H), 6.52 (J=8.5 Hz, 2H), 7.50 (J=8.5 Hz, 2H), 7.58 ppm (J=8.5 Hz, 2H)] attributable to hydroxyphenylglycine moieties.

Although the data presented are preliminary some information can be drawn. A-16686 is a complex of three closely-related antibiotics formed by polypeptide chains, containing aromatic moieties bearing hydroxyls and/or chlorine atoms, linked to the neutral sugar D-mannose.

Biological Activity

A-16686 possesses good activity against Gram-positive bacteria. Details are given in the accompanying paper[6].

The effect on macromolecular synthesis was tested by adding the antibiotic to exponentially growing cultures of *B. subtilis*. At a concentration ten times the MIC it gives a rapid suppression of cell wall synthesis (Fig. 7).

A-16686 is inactive on bacteria that lack a cell wall, such as the L-form of sensitive *S. aureus*. It does not show cytotoxic activity on cultured mammalian cells.

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* Recorded at 270 MHz in a 1:1 solution of DMF-d_7 and D_2O on a Bruker WH-270 Cryospectrometer (TMS δ=0.00 as internal reference). The attributions were based on the amino acid and sugar composition outlined above and from literature data.