

Antibiotic A/16686 factor A₂, the process for the preparation thereof and the co-produced antibiotic A/16686 factors A₁ and A₃

The present invention relates to an antibiotic substance arbitrarily designated as antibiotic A/16686 factor A₂, to the process for the preparation thereof and to the co-produced antibiotic A/16686 factors A₁ and A₃. The first object of the present invention is antibiotic A/16686 factor A₂, a chlorine containing antibiotic substance, in an essentially pure form. This antibiotic substance is produced by culturing a hitherto undescribed strain which has been characterized taxonomically as a novel species of the *Actinoplanes* genus.

A culture of this strain, which was isolated from a soil sample collected at Vaghalbod (India), has been deposited on January 30, 1979 with the permanent culture collection of ATCC (American Type Culture Collection—12301 Parklawn Drive, Rockville—Maryland 20852—U.S.A.), where it has been accorded the accession number ATCC 33076.

A copending application, now published in Great Britain with the number 2045231, describes an antibiotic substance named A/16686 which is recovered from the fermentation broth of *Actinoplanes* Sp. ATCC 33076.

Antibiotic A/16686 is therein characterized by a wide series of different chemico-physical parameters including the Rf values in a set of paper and thin layer chromatographic systems where this compound behaves as a true unitary product.

It has been found unexpectedly that antibiotic A/16686 actually comprises a mixture of three coproduced closely related antibiotics named antibiotic A/16686 factor A₁, antibiotic A/16686 factor A₂ and antibiotic A/16686 factor A₃.

Besides antibiotic A/16686 factor A₂ which is the major factor produced by this culture, other two minor individual antibiotic factors have been isolated and separated from a fermentation broth produced by the new *Actinoplanes* culture. These individual antibiotics are arbitrarily designated as antibiotic A/16686 factors A₁ and A₃. Antibiotic A/16686 factor A₂ is produced by culturing the strain *Actinoplanes* sp. ATCC 33076 under submerged aerobic fermentation conditions until a substantial level of antibiotic activity is produced. Antibiotic A/16686 factor A₂ is recovered as an antibiotic complex with factors A₁ and A₃ by extraction of both the broth and the mycelium with polar organic solvents, and is then separated from the A/16686 complex and isolated as an essentially pure individual antibiotic compound by chromatographic techniques such as HPLC, column chromatography and preparative thin layer chromatography. The same techniques are also useful for separating the co-produced factors A₁ and A₃.

Antibiotic A/16686 factor A₂, as well as the co-produced factors A₁ and A₃, have a basic character and are therefore capable of forming acid addition salts. It is intended that physiologically acceptable acid addition salts of antibiotic A/16686 factor A₂, as well as those of antibiotic A/16686 factors A₁ and A₃, are part of the present invention.

"Physiologically-acceptable" acid addition salts are salts which are also pharmaceutically acceptable, that is, salts in which the toxicity of the compound as a whole is not increased relative to the non-salt form. Representative and suitable acid addition salts include the addition salts with organic or inorganic acids such as hydrochloric, hydrobromic, sulfuric, phosphoric, nitric, tartaric, acetic, succinic, lactic, glutamic, methanesulfonic and the like acids.

A/16686 factors A₁, A₂, and A₃ free bases can be prepared from the corresponding acid addition salts, or *vice versa* A/16686 factor A₁, A₂, and A₃ as acid addition salts can be prepared from the corresponding free bases, according to procedures commonly employed in this field. The free bases of antibiotic A/16686 factor A₁, A₂, and A₃ are obtained from the corresponding acid addition salts, for instance, by treatment of a solution of the acid addition salt with barium hydroxide while the reaction of the free bases with the selected organic or inorganic acid in an inert solvent solution affords the corresponding acid addition salts.

For simplicity in discussions of utility, the term A/16686 compound is used herein to refer to a compound selected from the group consisting of A/16686 factors A₁, A₂, and A₃ and the corresponding non-toxic physiologically acceptable acid addition salts.

The A/16686 compounds inhibit *in vitro* the growth of certain pathogenic bacteria, especially gram-positive; moreover parenteral administration of A/16686 compounds gives a high degree of protection against experimental infections in mice. In addition the A/16686 compounds are useful in the prevention and treatment of dental caries.

As stated above, antibiotics A/16686 are produced by culturing a novel strain of the *Actinoplanes* genus named *Actinoplanes* sp. ATCC 33076.

The characteristics of this strain are given in the following paragraphs.

Morphology

The strain grows well on different media with an orange color of the substrate mycelium. It does not produce pigment. Aerial mycelium is always absent.

At microscopic examination of the vegetative mycelium reveals branched hyphae with a diameter of about 1 μ m. The sporangia form scantily only on potato-agar 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. The subspherical spores are motile (1.0—1.5 μm diameter). Analysis of the cell-wall components reveals meso-diaminopimelic acid and sugar pattern of type D (Lechevalier et al.—Chemical composition as a criterium in the classification of Actinomycetes. Adv. Applied Microbiology, 14, 1971. Academic Press, N.Y.).

Cultural characteristics

Table 1 reports the culture characteristics of *Actinoplanes* ATCC 33076 cultivated on various standard media suggested by Shirling and Gottlieb (Intern. J. System. Bact. 16, 313—340, 1966) and other media recommended by Waksman (The Actinomycetes, Vol. II—The Williams and Wilkins Co. 1961). The cultural characteristics were determined after 6 to 14 days of incubation at 30°C.

TABLE I

Cultural characteristics

The number of some of the culture media refers to those given by Shirling and Gottlieb in Methods for characterization of *Streptomyces* species—Intern. J. System. Bact. 16, 313—340, 1966.

	Culture media	Culture characteristics
20	Medium No. 2 (yeast extract-malt agar)	Abundant growth, wrinkled surface, light brown 12 H 12
	Medium No. 3 (oatmeal agar)	Scant growth, thin, light orange 9 B 6
25	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
30	Medium No. 7 (tyrosine agar)	Scant growth, smooth surface, brown 6 D 11
	Oatmeal agar (according to Waksman)	Abundant growth, wrinkled surface, orange to brown 12 C 10
	Hickey and Tresner's agar	Abundant growth, crusty surface, orange 11 G 8
35	Czapeck glucose agar	Moderate growth, crusty surface, orange 11 G 8
	Glucose asparagine agar	Scant growth, crusty surface, light orange 11 F 6
40	Nutrient agar	Moderate growth, smooth surface, orange 11 G 8
	Potato agar	Abundant growth, wrinkled surface, amber-brown 12 E 10
	Bennett's agar	Abundant growth, wrinkled surface, orange 11 G 8
45	Calcium malate agar	Moderate growth, smooth surface, light orange 10 C 6
	Skim milk agar	Abundant growth, wrinkled surface, orange 9 L 2
50	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
55	Agar	Very scant growth, smooth surface, hyaline
	Loeffler serum	Very scant growth, smooth surface, orange
60	Potato	Scant growth, crusty, light brown
	Gelatin	Scant growth, light orange
	Cellulose	Very scant growth, thin, hyaline

Letters and numbers refer to the color determined according to Maerz and Paul—A dictionary of color—McGraw Hill Inc., New York, 1950.

Carbon utilization

Table II reports the utilization of carbon sources examined according to the method of Pridham and Gottlieb (J. Bact. 56, 107, 1948).

TABLE II

	Carbon sources	Utilization
	Inositol	—
10	Fructose	+
	Rhamnose	+
	Mannitol	—
	Xylose	+
	Raffinose	—
15	Arabinose	+
	Cellulose	—
	Sucrose	+
	Glucose	+
	Mannose	+
20	Lactose	—
	Salicin	+

+ = positive utilization

25 — = no growth

Physiological characteristics

Table III reports the physiological characteristics of the strain.

TABLE III

	Test	Results
	Hydrolysis of starch	positive
35	H ₂ S formation	positive
	Tyrosine reaction	negative
	Casein hydrolysis	positive
	Solubilization of calcium malate	negative
40	Liquefaction of gelatine	positive
	coagulation	positive
	Litmus milk	
	peptonization	negative
45	Cellulose decomposition	negative

As in the case with other organisms, the characteristics of the A/16686 factor A₂ producing strain *Actinoplanes* sp. ATCC 33076, are subject to variation. For example, artificial variants and mutants of the ATCC 33076 strain may be obtained by treatment with various known mutagens such as ultraviolet rays, X rays, high-frequency waves, radioactive rays and chemicals. All natural and artificial variants and mutants which belong to this *Actinoplanes* species and produce antibiotic A/16686 factor A₂ may be used in this invention.

For producing the A/16686 antibiotics the strain *Actinoplanes* sp. ATCC 33076 is cultivated under aerobic conditions in an aqueous nutrient medium containing an assimilable source of carbon, an assimilable source of nitrogen and inorganic salts. Said culture medium can be anyone of a number of nutrient media usually employed in the fermentation art, however certain media are preferred. Thus, for instance, preferred carbon sources are glucose, fructose, mannose, sucrose and the like in various grades of purity. Preferred nitrogen sources are soybean meal, peptone, meat extract, yeast extract, tryptone, amino acids and the like. Among the inorganic salts which can be incorporated in the culture media are the customary soluble salts capable of yielding sodium, potassium, iron, zinc, cobalt, magnesium, calcium, ammonium, chloride, carbonate, sulfate, nitrate and like ions. Ordinarily the antibiotic-producing strain is precultured in a shake flask, then the culture is used to inoculate jar fermentors for production of substantial quantities of the A/16686 antibiotics. The medium used for

the preculture can be the same as that employed for larger fermentations, but other media can also be employed.

The A/16686-producing strain can be grown at temperatures between about 20°C and about 37°C and preferably at temperatures of about 28—30°C.

5 During the fermentation, antibiotic production can be followed by testing samples of the broth or of extracts of the mycelial solids for antibiotic activity.

Organisms known to be sensitive to the A/16686 antibiotics are useful for this purpose. One especially useful assay organism is *Sarcina lutea* ATCC 9341. The bioassay is conveniently performed by the agar diffusion method on agar plates. Maximum production of antibiotic activity generally occurs between about the third and the fifth days. The antibiotics produced during fermentation of the strain 10 *Actinoplanes* sp. ATCC 33076 are found mainly in the mycelial mass. A preferred method of recovering the A/16686 antibiotics is, therefore, by extraction of the separated mycelium.

Extraction of the mycelial mass is best accomplished with methanol, but other lower alkanols and acetone are also suitable. The A/16686 antibiotics are recovered from the extracting solvent by routine 15 procedure to give a mixture of the A/16686 antibiotics, the A/16686 complex. The A/16686 complex is further purified, and then the individual factors are separated from each other. Separation of the A/16686 complex into the single components may be achieved by a variety of recognized methods which essentially involve chromatographic procedures. For optimum separation of factors, reverse phase HPLC is preferred. In such HPLC separation, a preferred column is μ -Bondapack® C₁₈ and 20 preferred mobile phases are mixtures of aqueous HCOONH₄ and CH₃CN in variable ratios.

For large scale separation of antibiotic A/16686 factor A₂ column chromatography is preferably employed. In such column separation a preferred adsorbent is Amberlite® XAD and preferred solvent systems are mixtures of water and acetonitrile and mixtures of ammonium formate and acetonitrile. Separation of minor factors A₁ and A₃ by column chromatography may be carried out but it requires 25 subsequent column separation of enriched fractions. Again Amberlite XAD® is a preferred adsorbent and mixtures of water and acetonitrile and mixture of ammonium formate and acetonitrile are the preferred solvent systems.

A/16686 Factor A₂

30 A/16686 factor A₂ is a white amorphous powder which melts with decomposition at about 210—220°C.

A/16686 factor A₂ is soluble in water, dimethylformamide, aqueous methanol, 0.1 N HCl; it is poorly soluble in absolute ethanol and in n-butanol; it precipitates from the aqueous solution by the 35 addition of a solution saturated with NaHCO₃.

Elemental analysis of A/16686 factor A₂ previously dried at about 140°C under inert atmosphere indicates the following approximate percentage composition (average); carbon, 54.57%, hydrogen, 6.19%, nitrogen, 10.88%.

Chlorine analysis gives the following percentage: chlorine 1.37%. The infrared absorption 40 spectrum of A/16686 factor A₂ in nujol is shown in figure 1 of the accompanying drawings. The following absorption maxima are observed: 3290, 2930 and 2860 (nujol), 1765, 1635, 1510, 1455 and 1375 (nujol), 1240, 1175, 1130, 1060, 1015, 975, 840 and 815 cm⁻¹.

The ultraviolet absorption spectrum of A/16686 factor A₂, which is given in figure 2 of the accompanying drawings, exhibits the following absorption maxima:

45 a) in neutral methanol:

234 nm ($E_{1\%}^{1\text{cm}}=206$)

268 nm ($E_{1\%}^{1\text{cm}}=114$)

50 b) in methanol containing 0.1 N HCl

233 nm ($E_{1\%}^{1\text{cm}}=192$)

55 271 nm ($E_{1\%}^{1\text{cm}}=93$)

c) in methanol containing 0.1 N NaOH

251 nm ($E_{1\%}^{1\text{cm}}=275$)

60 ~300 nm (shoulder)

A/16686 factor A₂ gives the following signals in 270 MHz ¹H NMR spectrum, figure 3, registered in DMF-d₇/D₂O 1:1 (v/v) solution (TMS as internal standard δ 0.00 ppm) on a Pulse Fourier Transform 65 Bruker WH 270 cryospectrometer:

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- (i) a group of signals at 0.5—3 ppm corresponding to about 51 aliphatic protons, in particular doublets centred approximately at 1.53 ppm ($J=7$ Hz) 1.20 ppm ($J=6$ Hz) and 1.07 ppm ($J=5$ Hz).
(ii) a group of signals at 3—6.5 ppm corresponding to about 43 protons of olefinic type or protons of carbons linked to hetero atoms, in particular a singlet approximately at 5.62 ppm.
5 (iii) a group of signals at 6.5—8 ppm corresponding to about 39 protons of aromatic type, in particular doublets centred approximately at 7.60 ppm ($J=8$ Hz), at 7.49 ppm ($J=8$ Hz), and at 6.46 ppm ($J=8$ Hz).

The singlets at 8, 2.85 and 3.01 ppm and the singlet at 4.73 ppm correspond respectively to
10 DMF and H₂O present in the solvent mixture.

A/16686 factor A_2 has a specific rotation $[\alpha]_D^{20}$, of $+73 \pm 4^\circ$ ($c=0.49$, H₂O).

The R_f values of A/16686 factor A_2 in various paper-chromatographic systems, using *B. subtilis* ATCC 6633 as a detection organism, are given in the following table:

15
20
25
30
35
40
45
50
55
60
65

TABLE IV
Chromatographic behaviour of A/16686 factor A_2
(Whatman No. 1 paper)*

Elution system (v/v/v)	R_f value
1) n-butanol saturated with Sørensen buffer pH 6.0	0.00
2) n-butanol saturated with water containing 2% of p-toluenesulfonic acid	0.00
3) n-butanol saturated with water containing 2% of ammonium hydroxide	0.00
4) Sørensen buffer, pH 6.0, saturated with n-butanol	0.05
5) n-butanol:methanol:water:4:1:2	0.48
6) ethyl acetate saturated with water	0.00
7) n-butanol:acetic acid: water 2:1:1	0.44

* Descending chromatography

R_m: 40 cm

35 Amounts: 50 μ g of the compound dissolved in H₂O:CH₃CN 1:1 (v/v) (2 mg/ml)

The R_f value of A/16686 factor A_2 in various thin-layer chromatographic systems are listed in the following table (the conditions are indicated below the table):

40
45
50
55
60
65

TABLE V
Elution system (v/v/v)

Elution system (v/v/v)	R_f value
1) aqueous 2.5% HCOONH ₄ :CH ₃ CN (65:35)*	0.36
2) n-butanol:acetic acid: water 4:2:5	0.80
3) n-butanol:acetic acid: water 4:2:5	0.52
4) n-propanol:n-butanol: 1N NH ₄ OH 2:3:4 (upper phase)	0.12
5) n-butanol:acetic acid:water 4:1:5	0.15

50 R_m: about 140 mm

Amounts: 2—5 μ l of a solution (1 mg/ml) in CH₃CN: H₂O 1:1 (v/v)

Visualization: a) Iodine vapour b) U.V. light at 254 nm. 1,2-silanised Silicagel 60 F₂₅₄ plates (Merck): visualization a,b; 3,4,5-Silicagel 60 F₂₅₄ plates (Merck): visualization a. (*Internal standards: Caffeine R_f 0.60; Cortisone R_f 0.35; Dexamethasone R_f 0.30).

A/16686 factor A_2 shows the following characteristic reactions:

Ninhydrin (3% ethanolic solution)	positive
Molish	positive
Biuret	positive
Millon	negative
1% FeCl ₃ —1% K ₃ Fe(CN) ₆ aqueous	green color
KMnO ₄ (acidic)	positive
H ₂ SO ₄ conc.	negative

Amino acid analysis of A/16686 factor A₂ after acid hydrolysis with 6N hydrochloric acid at 110°C for 6 hours, revealed the presence of at least the following amino acids: alanine, leucine, glycine, aspartic acid, phenylalanine, ornithine p-hydroxyphenylglycine, and hydroxy, chloro-substituted phenylglycine. Analysis of an acid hydrolyzate of A/16686 factor A₂ after 2 hours in 2N H₂SO₄ at 100°C, shows the presence of the neutral carbohydrate D-mannose.

HPLC analysis of antibiotic A/16686 factor A₂ using a μ -Bondapack® C₁₈ column (3.9 mm ID x 300 mm) and a mixture HCOONH₄ 0.025 M/CH₃CN 60/40 (v/v) as the mobile phase with a flow rate of 2 ml/min, showed that the compound has a retention time (t_R) of 4' 99/100" (Internal standards: Anthracene t_R 35' 18/100"; α -Nitronaphthalene t_R 11' 14/100"; Toluene t_R 8' 64/100").

A/16686 Factor A₁

A/16686 factor A₁ is a white amorphous powder which melts with decomposition at about 210—220°C. It is very soluble in water, dimethylformamide, and aqueous methanol; is soluble in methanol, and ethanol; but is insoluble in ethyl ether, petroleum ether, and benzene. Elemental analysis of A/16686 factor A₁ previously dried at about 140°C under inert atmosphere, indicates the following approximate percentage composition (average): carbon 50.31% hydrogen, 6.00% nitrogen, 9.92%, residue 6.07%.

Chlorine analysis of A/16686 factor A₁ gives the following results: chlorine (total content), 1.61%, chlorine ion, 0.90%.

The infrared absorption spectrum of A/16686 factor A₁ in nujol is shown in figure 4 of the accompanying drawings.

The following absorption maxima are observed: 3290, 2930 and 2860 (nujol), 1765, 1630, 1510, 1455 and 1375 (nujol), 1260, 1235, 1175, 1150, 1130, 1060, 1015, 975, 840, and 810 cm⁻¹.

The ultraviolet absorption spectrum of A/16686 factor A₁ which is shown in figure 5 of the accompanying drawings exhibits the following absorption maxima:

a) in neutral methanol:

233 nm (E_{1%¹cm} = 202)

266 nm (E_{1%¹cm} = 106)

b) in methanol containing 0.1N HCl:

232 nm (E_{1%¹cm} = 219)

270 nm (E_{1%¹cm} = 110)

c) in methanol containing 0.1N NaOH

251 nm (E_{1%¹cm} = 264)

~290 nm (shoulder)

A/16686 factor A₁ has a specific rotation, $[\alpha]_D^{20}$, of $+57 \pm 4^\circ$ (c=0.51, H₂O).

The R_f values of A/16686 factor A₁ in various paper-chromatographic systems, using *B. subtilis* ATCC 6633 as a detection organism, are given in the following table.

TABLE VI
Chromatographic behaviour of A/16686 factor A₁
(Whatman No. 1 paper)*

Elution system (v/v/v)	R _f value
1) n-butanol saturated with Sørensen buffer pH 6.0	0.00
2) n-butanol saturated with water containing 2% of p-toluenesulfonic acid	0.00
3) n-butanol saturated with water containing 2% of ammonium hydroxide	0.00
4) Sørensen buffer, pH 6.0, saturated with n-butanol	0.05
5) n-butanol:methanol:water 4:1:2	0.48
6) ethyl acetate saturated with water	0.00
7) n-butanol:acetic acid:water 2:1:1	0.44

* Descending chromatography

R_m: 40 cm

Amounts: 50 µg of the compound dissolved in H₂O: CH₃CN 1:1 v/v (2 mg/ml)

The R_f values of A/16686 factor A₁ in various thin-layer chromatographic systems are listed in the following table (the conditions are indicated below the table):

Elution system (v/v/v)	R _f value
1) Aqueous 2.5% HCOONH ₄ : CH ₃ CN 65:35*	0.40
2) n-butanol:acetic acid:water 4:2:5	0.80
3) n-butanol:acetic acid:water 4:2:5	0.52
4) n-propanol:n-butanol: 1N NH ₄ OH 2:3:4 (upper phase)	0.12
5) n-butanol:acetic acid:water 4:1:5	0.15

R_m: about 140 mm

Amounts: 2—5 µl of a solution (1 mg/ml) in CH₃CN: H₂O 1:1 (v/v)

Visualization: a) Iodine vapour b) UV-light at 254 nm

1,2-Silanised silicagel 60 F₂₅₄ plates (Merck): visualization a,b; 3,4,5-silicagel 60 F₂₅₄ Merck: visualization a. (*Internal standards: Caffeine R_f 0.60; Cortisone R_f 0.35; dexamethasone R_f 0.30).

Amino-acid analysis of A/16686 factor A₁, after acidic hydrolysis in 6N hydrochloric acid at 110°C for 6 hours, indicates the presence of at least the following aminoacids: alanine, leucine, glycine, aspartic acid, phenylalanine, ornithine, p-hydroxy-phenylglycine, and hydroxy, chloro-substituted phenylglycine.

Analysis of an acid hydrolyzate of A/16686 factor A₁ after 2 hours in 2N H₂SO₄ at 100°C shows the presence of the neutral carbohydrate D-mannose.

HPLC analysis of antibiotic A/16686 factor A₁ using a µ-Bondapack® C₁₈ column (3.9 mm ID×300 mm) and a mixture HCOONH₄ 0.025 M/CH₃CN 60/40 (v/v) as the mobile phase at a flow rate of 2 ml/min gave a t_R of 3' 84/100" (Internal standards: Anthracene t_R 35' 18/100"; α-Nitro-naphthalene t_R 11' 14/100"; Toluene 8' 64/100").

A/16686 Factor A₃

A/16686 factor A₃ is a white amorphous powder which melts with decomposition at about 220°C. It is very soluble in water, dimethylformamide and aqueous methanol; it is soluble in methanol, and ethanol but it is insoluble in ethyl ether, petroleum ether, and benzene.

Elemental analysis of A/16686 factor A₃ previously dried at about 140°C under inert atmosphere indicates the following approximate percentage composition (average) carbon, 48.41% hydrogen 5.84%, nitrogen 9.09%; residue 9.1%. Chlorine analysis gives the following results: chlorine (total content), 1.68% chlorine ions, 1.16%. The infrared absorption spectrum of A/16686 factor A₃ in nujol is shown in figure 6 of the accompanying drawings. The following absorption maxima are observed: 3290, 2930 and 2860 (nujol), 1765, 1635, 1510, 1455 and 1375 (nujol), 1260, 1240, 1175, 1100, 1060, 1020, 975, 840, and 805 cm⁻¹.

The ultraviolet absorption spectrum of A/16686 factor A₃ which is given in figure 7 of the accompanying drawings exhibits the following absorption maxima.

17

a) in neutral methanol

234 nm ($E_{1\text{ cm}}^{1\%} = 147$)267 nm ($E_{1\text{ cm}}^{1\%} = 74$)

b) in methanol containing 0.1N HCl

232 nm ($E_{1\text{ cm}}^{1\%} = 132$)270 nm ($E_{1\text{ cm}}^{1\%} = 76$)

c) in methanol containing 0.1N NaOH

250 nm ($E_{1\text{ cm}}^{1\%} = 202$)

~295 nm (shoulder)

A/16686 factor A_3 has a specific rotation, $[\alpha]_D^{20}$, of $+50 \pm 4^\circ$ ($c=0.48$, HCl N/100).The R_f values of A/16686 factor A_3 in various paper-chromatographic systems, using *B. subtilis* ATCC 6633, as a detection organism, are given in the following table:

TABLE VIII
Chromatographic behaviour of A/16686 factor A_3
(Whatman No. 1 paper)*

Elution system (v/v/v)	R_f value
1) n-butanol saturated with Sørensen buffer pH 6.0	0.00
2) n-butanol saturated with water containing 2% of p-toluenesulfonic acid	0.00
3) n-butanol saturated with water containing 2% of ammonium hydroxide	0.00
4) Sørensen buffer, pH 6.0 saturated with n-butanol	0.05
5) n-butanol:methanol:water: 4:1:2	0.48
6) ethyl acetate saturated with water	0.00
7) n-butanol:acetic acid:water 2:1:1	0.44

* Descending chromatography

Rm: 40 cm

Amounts: 50 μ g of the compound dissolved in H_2O : CH_3CN 1:1 (v/v) (2 mg/ml)The R_f value of A/16686 factor A_3 in various thin-layer chromatographic systems are listed in the following table (the conditions are indicated below the table):

TABLE IX
Elution system (v/v/v)

Elution system (v/v/v)	R_f value
1) aqueous 2.5% $HCOONH_4$: CH_3CN (65:35)*	0.32
2) n-butanol:acetic acid:water 4:2:5	0.80
3) n-butanol:acetic acid:water 4:2:5	0.52
4) n-propanol:n-butanol: 1N NH_4OH 2:3:4 (upper phase)	0.12
5) n-butanol:acetic acid:water 4:1:5	0.15

Rm: about 140 mm

Amounts: 2—5 μ l of a solution (1 mg/ml) in CH_3CN : H_2O 1:1

Visualization: a) Iodine vapour b) U.V. light at 254 nm.

1,2-Silanised silicagel 60 F₂₅₄ plates (Merck): visualization a, b; 3,4,5-silicagel 60 F₂₅₄ plates (Merck): visualization a. (*Internal standards: Caffeine R_f 0.60; Cortisone R_f 0.35; dexamethasone R_f 0.30).

Amino-acid analysis of A/16686 factor A₃ after acid hydrolysis in 6N hydrochloric acid at 110°C for 6 hours revealed the presence of at least the following aminoacids: alanine, leucine, glycine, aspartic acid, phenylalanine, ornithine, p-hydroxyphenylglycine, and hydroxy, chloro substituted phenylglycine.

Analysis of an acid hydrolyzate of A/16686 factor A₃ after 2 hours in 2N H₂SO₄ at 100°C shows the presence of the neutral carbohydrate D-mannose.

HPLC analysis of antibiotic A/16686 factor A₃ using a μ -Bondapack® C₁₈ column (3.9 mm ID x 300 mm) and a mixture HCOONH₄ 0.025 M/CH₃CN 60/40 (v/v) as the mobile phase at a flow rate of 2 ml/min gave a t_R of 6' 84/100". (Internal standards: anthracene t_R 35' 18/100"; α -nitronaphthalene t_R 11' 14/100"; toluene t_R 8' 64/100").

Antibiotic A/16686 factor A₂, as well as factor A₁ and A₃ are antimicrobial agents and are especially active against gram-positive microorganisms. In particular, the *in vitro* activity spectrum of the A/16686 antibiotics is summarized in the following Table:

TABLE X

Organisms	MIC (μ g/ml)		
	Factor A ₁	Factor A ₂	Factor A ₃
S. aureus ATCC 6538	0.1	0.1	1.6
S. aureus Tour (inoculum 10 ³ /ml)	0.2	0.2	1.6
S. aureus Tour (inoculum 10 ⁶ /ml)	0.4	0.8	3.1
S. aureus Tour (+30% bovine serum)	0.4	0.8	1.6
S. pyogenes C 203 SKF 13400	0.012	0.012	0.012
S. pneumoniae Felton UC 41	0.025	0.025	0.025
S. foecium ATCC 10541	0.1	0.05	0.1
E. coli SKF 12140	>100	>100	>100

The A/16686 antibiotics have also been found to possess a high order of activity *in vivo* against various pathogenic organisms. The effectiveness of A/16686 antibiotics is readily apparent from Table XI which gives the ED₅₀ values in mice against two different microorganisms.

TABLE XI

	ED ₅₀ mg/ml day s.c.	
	S. pyogenes C 203 SKF 13400	S. pneumoniae Felton UC 41
A/16686 factor A ₁	0.041 (0.012—0.064)	0.15 (0.17—0.14)
A/16686 factor A ₂	0.048 (0.026—0.070)	0.18 (0.20—0.16)
A/16686 factor A ₃	0.14 (0.096—0.21)	0.41 (0.46—0.36)

The A/16686 compounds also inhibit the growth of microorganisms which contribute to the development of periodontal diseases. This important property of the A/16686 compounds was assessed by means of tests using an artificial *Streptococcus mutans* ATCC 25175 plaque system. In these experiments the A/16686 compounds inhibited plaque formation at very low concentration levels. The A/16686 compounds may therefore be employed for the treatment of diseases due to pathogenic organisms susceptible to them. For instance, in the treatment of Streptococcal or Staphylococcal infections or for preventing and treating the dissolution and disintegration of the enamel and dentin of the teeth due to dental caries.

In such treatments the A/16686 compounds may be employed as free bases or as the corresponding non-toxic physiologically acceptable acid addition salts. Moreover they may be employed as single factors or, considering the similarity of their activity pattern, also in the form of mixtures of two or all of the three factors in any proportion.

In order to illustrate more fully the operation of this invention, the following examples are provided.

A

Example 1

Fermentation of the strain *Actinoplanes* sp. ATCC 33076

A culture of *Actinoplanes* sp. ATCC 33076 is precultured by growing the strain in shake-flasks containing 100 ml each of an aqueous nutrient medium having the following composition (in g/l)

5	meat extract	3 g/l
	yeast extract	5 g/l
	tryptone	5 g/l
	soluble starch	24 g/l
10	glucose	1 g/l
	CaCO ₃	4 g/l

The flasks are shaken for about 96 hours at 28—30°C and then are poured into a jar fermentor containing 4 l of the same vegetative medium and precultured for 48 hours at the same temperature. 3 l of this preculture are then used to inoculate a larger jar fermentor containing 30 l of the same nutrient medium seen above and aerobically precultured for 24 hours at 28°C. Finally, 20 l of this preculture are used to inoculate a tank fermentor containing 200 l of an aqueous nutrient medium having the following composition

20	meat extract	4 g/l
	peptone	4 g/l
	yeast extract	1 g/l
	sodium chloride	2.5 g/l
	soybean meal	10 g/l
25	glucose	25 g/l
	CaCO ₃	5 g/l

The fermentation batch is incubated aerobically under stirring at 28—30°C. At intervals, the antibiotic activity is assayed microbiologically by the agar diffusion method using *Sarcina lutea* ATCC 9341 as the test organism. The maximum activity is reached after 72 to 120 hours of fermentation.

Example 2

Separation of the antibiotic A/16686 complex

Whole fermentation broth (170 l) prepared as described in Example 1 is cooled to 10°C and brought to pH 3.5 by means of 18% HCl. The resulting acidic broth is filtered using a filter aid (Clarcel Flow-Ma), and the mycelial cake is washed with water.

Methanol (30 l) is used to extract the mycelial mass which, after filtration, is extracted again with a mixture methanol/water (30 l of methanol plus 5 l of water). The exhausted mycelium is discarded and the two methanol extracts are concentrated under vacuum at a temperature lower than 40°C to yield an aqueous concentrate (6 l). This aqueous concentrate is extracted with three portions, 10 l each, of n-butanol, which are combined and concentrated to a small volume under vacuum.

This concentrate is added to petroleum ether and the resulting precipitate is separated by decantation and added to a further amount of petroleum ether. The precipitate is separated by filtration and dried under vacuum at room temperature to give 80 g of antibiotic A/16686 complex as a raw material having a M.I.C. against *S. pneumoniae* UC 41 of 0.1 µg/ml.

Example 3

Purification of the antibiotic A/16686 complex

a) 47.7 g of the raw antibiotic A/16686 complex obtained in Example 2 are treated with 1.4 l of a chloroform:ethanol:water mixture (4:7:2) (v/v/v) and the oily product which forms is separated from the solution by decantation. Further 70 ml of the above mixture are then added to the oily product and the separation is repeated. By treatment of the oily product with water (440 ml), it solidifies and is separated by centrifugation or filtration:

The solid, which is separated, is suspended in water (170 ml), dissolved by addition of methanol (400 ml) and filtered. The pH of the aqueous methanol solution is brought to 3.5 by the addition of 1N HCl, then the solvents are stripped under vacuum by adding n-butanol at a temperature never higher than 35°C, to give a butanol concentrate of about 50 ml. By addition of diethyl ether (500 ml) a precipitate forms which is separated by filtration and dried under vacuum at room temperature yielding 1.012 g of rather pure antibiotic A/16686 complex having a M.I.C. on *S. pyogenes* of 0.025 µg/ml.

b) 1.58 g of antibiotic A/16686 complex obtained as described before are dissolved in 100 ml of a mixture acetonitrile: H₂O 1:1 (v/v) and the resulting solution is applied to a column containing 430 g of Silicagel 60 (Merck 0.06—0.2 mm) prepared in the same mixture. The column is developed first using the same acetonitrile-water mixture and collecting 70 fractions of 20 ml each, and then using acetonitrile: N/100 HCl 1:1 (v/v) and collecting further 290 fractions, of 20 ml each.

Elution of the column is monitored by thin layer chromatography on 60 F₂₅₄ Silicagel plates and by assaying fractions against *Sarcina lutea*.

Fractions 130 to 265 are combined and the solvents are stripped under vacuum with n-butanol to give a n-butanol concentrate of 20 ml. This residual volume is poured into a large amount of ethyl ether and the precipitate which form is separated by filtration and dried under vacuum at room temperature over P₂O₅ yielding 1.015 g of antibiotic A/16686 complex.

c) 0.67 g of the above substance are dissolved in 24 ml of water and 76 ml of methanol. The resulting solution is applied to a 3.0x62.0 cm column containing 220 g of Sephadex LH-20, prepared in methanol:water 7:3 (v/v). The column is developed with the same mixture collecting 10 ml fractions. Fractions containing antibiotic A/16686 complex are combined and the solvents are stripped under vacuum at a temperature lower than 35°C with n-butanol to a residual butanol volume of about 10 ml. This solution is added to ethyl ether to precipitate pure antibiotic A/16686 complex. The precipitate is separated by filtration washed with ethyl ether and dried under vacuum over P₂O₅ at room temperature yielding 0.26 g of pure antibiotic A/16686 complex.

The antibiotic A/16686 complex thus obtained is a white, crystalline material slightly hygroscopic which melts at 224—226°C. It is very soluble in water and dimethylformamide, soluble in methanol, ethanol, propanol and butanol, but is insoluble in ethyl ether, petroleum ether and benzene. Elemental analysis of the antibiotic A/16686 complex thus obtained, previously dried at 140°C under inert atmosphere, indicates the following approximate percentage composition (as an average of several analyses): carbon 51.73%; hydrogen 6.34%; nitrogen 9.96; residue 1%.

Chlorine analysis gives the following results: chlorine (total content) 5.48%; chlorine ions 4.74%.

The infrared absorption spectrum in nujol exhibits the following absorption maxima (in cm⁻¹): 3290, 3070, 2930 and 2860 (nujol), 1765, 1630, 1510, 1455e 1375 (nujol), 1230, 1175, 1130, 1065, 1030, 1015, 980, 840 and 820.

The ultraviolet absorption spectrum of the antibiotic A/16686 complex obtained as described in example 3 exhibits the following absorption maxima:

a) in methanol

232 nm ($E_{1\text{ cm}}^{1\%} = 178$)

265 nm ($E_{1\text{ cm}}^{1\%} = 107$)

b) in methanol containing 0.1N HCl

231 nm ($E_{1\text{ cm}}^{1\%} = 167$)

270 nm ($E_{1\text{ cm}}^{1\%} = 96$)

c) in methanol containing 0.1N NaOH

250 nm ($E_{1\text{ cm}}^{1\%} = 232$)

295 nm (shoulder)

d) in methanol containing pH 7.38 buffer

231 nm ($E_{1\text{ cm}}^{1\%} = 167$)

270 nm ($E_{1\text{ cm}}^{1\%} = 96$)

It has a specific rotation, $[\alpha]_D^{24}$, of +49.7° ($c=0.43\%$ in DMF).

The R_f values of antibiotic A/16686 complex in various paper-chromatographic systems, using *S. aureus* ATCC 6538 as a detection organism, are given in the following Table:

TABLE XII
Chromatographic behaviour (Whatman No. 1 paper)*
of antibiotic A/16686 complex

Elution system	R _f value
1) <i>n</i> -butanol saturated with Sørensen buffer pH 6.0	0.00
2) <i>n</i> -butanol saturated with water containing 2% of <i>p</i> -toluenesulfonic acid	0.00
3) <i>n</i> -butanol saturated with water containing 2% ammonium hydroxide	0.00
4) Sørensen buffer, pH 6.0, saturated with <i>n</i> -butanol	0.05
5) <i>n</i> -butanol:methanol:water 4:1:2	0.43
6) ethyl acetate saturated with water	0.00
7) <i>n</i> -butanol:acetic acid:water 2:1:1	0.52
8) <i>n</i> -butanol:pyridine:water 4:3:7	0.87

* Descending chromatography

R_m: 40 cm

Amounts: 20 µg of the compound dissolved in water methanol (2 mg/ml)

The R_f values of antibiotic A/16686 complex in various thin-layer chromatographic systems are listed in the following table (the conditions are indicated below the table):

TABLE XIII
Elution systems (v/v/v) R_f values

Elution systems (v/v/v)	R _f values
1) <i>n</i> -propanol: <i>n</i> -butanol:N ammonium hydroxide 2:3:4 (upper phase)	0.15
2) <i>n</i> -butanol:acetic acid:water 4:2:5	0.61
3) <i>n</i> -butanol:ethanol:0.1 N hydrochloric acid	0.61
4) chloroform:ethanol:10% acetic acid 4:7:2	0.00
5) <i>n</i> -butanol:acetic acid:water 4:1:5	0.17
6) methanol:10% aqueous ammonium acetate:10% ammonium hydroxide 10:9:1	0.52
7) <i>n</i> -butanol:pyridine:water:acetic acid 6:4:3:1	0.45
8) methanol:10% aqueous ammonium acetate:10% ammonium hydroxide 10:9:1	0.11
9) 0.25 M aqueous NaH ₂ PO ₄ :acetonitrile 1:1	0.68
10) methanol:10% aqueous ammonium acetate 1:1	0.65
11) <i>n</i> -butanol:acetic acid:water 4:2:5	0.77

R_m: about 140 mm

Amounts: 2—5 µl of a solution (1 mg/ml) of the compound in acetonitrile-water 1:1 (v/v).

Visualization: a) bioautography on agar plates seeded with *B. subtilis* ATCC 6633; b) carbonisation by heating with α -naphtholsulfuric acid; c) Iodine vapour; d) chlorine-toluidine reagent; e) UV-light at 254 nm.

1 to 7—Silicagel 60 F₂₅₄ plates (Merck); Visualization a, b, c, d, e.

8, 9—Silicagel 60 F₂₅₄ silanised (Merck); Visualization e.

10, 11—Cellulose F plates (Merck); Visualization a, e.

HPLC analysis of antibiotic A/16686 complex, isolated and purified as described in example 3, revealed the presence of three factors named antibiotic A/16686 factor A₁, A₂, and A₃ of which the major factor, amounting to approximately 70—87%, is A/16686 factor A₂. More particularly, the results of the HPLC analysis and the conditions in which it was performed are illustrated in the following table (Toluene, α -nitronaphthalene and anthracene have been used as internal standards):

TABLE XIV

Peak No.	t_R
A ₁	3' 84/100"
A ₂	4' 99/100"
A ₃	6' 84/100"
Toluene	8' 64/100"
α -Nitronaphthalene	11' 14/100"
Anthracene	35' 18/100"

10 Column: μ Bondapack® C₁₈ (3.9 mm IDx300 mm)
 Mobile phase: HCOONH₄ 0.025 M: CH₃CN 60:40 (v/v)
 Flow: 2 ml/min
 Pressure: 2000 psi
 Detector: UV 254 nm

15 Example 4

Separation of antibiotic A/16686 factors A₁, A₂, and A₃

A/16686 complex (169 mg) obtained as described in example 3 is dissolved in 0.01 N hydrochloric acid (6.5 ml) and distilled water (10.4 ml). By reverse phase HPLC [column: μ -Bondapack® C₁₈ (7.8 mm IDx300 mm)—flow: 4 ml/min—pressure: 2000 psi—mobile phase: HCOONH₄ 0.025 M: CH₃CN 65:35 (v/v)] with repeated injections of 1 or 2 ml of the above solution, fractions containing the single factors are collected and further checked by means of analytical HPLC. The fractions are concentrated under vacuum at a temperature lower than 35°C by adding butanol in order to reduce foam formation. The residual solutions are lyophilized and the obtained solids are separately taken up with distilled water and lyophilized again yielding antibiotic A/16686 factors A₁ (10 mg), A₂ (95 mg), and A₃ (12 mg). By lyophilizing a hydrochloric solution of A/16686 factor A₂ obtained as above, the corresponding hydrochloride is obtained as an amorphous white powder which decomposes at 250°C.

30 Example 5

Separation of antibiotic A/16686 factor A₂

Antibiotic A/16686 complex (100 mg), obtained as described in example 3b) (titre: ~40%) is dissolved in 0.1N hydrochloric acid (15 ml). The resulting solution is applied to a column of Amberlite® XAD-2 (136 ml, height of the bed: 60 cm), previously washed with CH₃CN and with distilled water. The column is developed with a linear gradient 0 to 34% CH₃CN/water (v/v) elution, collecting 250 10-ml fractions.

Fractions 185 to 225 which contain A/16686 factor A₂ alone, are combined and concentrated under vacuum at a temperature lower than 35°C by adding butanol in order to reduce foam formation. The residual solution is lyophilized yielding antibiotic A/16686 factor A₂ (6.7 mg).

40 Example 6

Separation of antibiotic A/16686 factor A₂

Antibiotic A/16686 complex (100 mg) obtained as described in example 3a) (titre: 53.5%) is dissolved in 10 ml of CH₃CN/0.025 M aqueous HCOONH₄ 27/73 (v/v). The resulting solution is applied to a column of Amberlite® XAD-2 (200 ml—height of the bed: 52 cm), previously washed with 0.025 M HCOONH₄ first and then with CH₃CN/0.025 M HCOONH₄ 27/73 (v/v). The column is developed with a linear gradient 27 to 40% CH₃CN/0.025 M HCOONH₄ collecting 10 ml fractions up to 400 ml and then 6 ml fractions up to a total volume of eluent of 2 liters. The eluted fractions are checked by analytical HPLC, combined according to factor content and titrated by HPLC, giving the following results:

Fractions	% A/16686 factor A ₁	% A/16686 factor A ₂	% A/16686 factor A ₃
31—41	90.1	9.9	0
42—50	53.9	46.1	0
51—59	17.9	82.1	0
60—65	4.4	93.7	1.9
66—75	0	94.2	5.8
76—80	0	83.7	16.3
81—99	0	46.1	53.8
100—110	0	6.7	93.3

23

Fractions 60 to 75 which contain 94% pure Antibiotic A/16686 factor A₂ are concentrated under vacuum at a temperature lower than 35°C by adding butanol and the residual solution is lyophilized, taken up with distilled water and lyophilized again yielding 19.7 mg of A/16686 factor A₂.

5 Example 7

Separation of antibiotic A/16686 factor A₁ and A₂

A solution of antibiotic A/16686 complex (650 mg) obtained as described in Example 3a) (titre 53.5%) is dissolved in a mixture CH₃CN/HCOONH₄ 0.025 M 26/74 (v/v) and the obtained solution is percolated through a column of Amberlite XAD-2® (1900 ml-height of the bed: 130 cm) previously
10 washed with the same solvent mixture, at a flow of ~9 ml/min.

A 100-ml volume is collected, then the column is developed with the same solvent system at the same flow collecting 300 ml fractions up to 18.3 l and then 500 ml fractions up to a total volume of 21.3 l. The eluted fractions are checked by analytical HPLC, combined according to factor content and
15 titrated by HPLC giving the following results:

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(24)

A/16686 complex as obtained in Ex. 3a)	%A/16686 factor A ₁ *	%A/16686 factor A ₂ *	%A/16686 factor A ₃ *	A/16686 factor A ₁ ** (mg)	A/16686 factor A ₂ ** (mg)	A/16686 factor A ₃ ** (mg)	Total (mg)
	17.4	71.0	11.6	60.5	246.4	40.3	347
Fractions							
19-24	100	0	0	14.4	—	—	14.4
25-26	80	20	0	8.2	2.1	—	10.3
27	73.8	26.2	0	3.6	1.3	—	4.9
28-31	42.2	57.8	0	9.5	13.1	—	22.6
32-35	15	85	0	4.8	27.2	—	32
36-38	3.4	96.6	0	1.2	33.6	—	34.8
39-48	0	100	0	—	107.1	—	107.1
49-58	0	92.7	7.3	—	40.8	3.2	44.0
59-61	0	70.8	29.2	—	4.0	1.7	5.7
62-63	0	43.5	56.5	—	2.6	3.4	6.0
64-67	0	10.6	89.4	—	3.3	28	31.3

* calculated on the basis of the relative peaks' height.

** determined by comparing the height of the relative peak with that of a standard sample of A/16686 factor A₂.



(25)

Claims

1. Antibiotic A/16686 factor A₂ as an essentially pure individual antibiotic compound, and its non-toxic physiologically-acceptable acid addition salts, characterized in the form of free base, by:

A) being a white amorphous powder which melts with decomposition at about 210—220°C.

B) being very soluble in water, dimethylformamide, aqueous methanol, and 0.1N HCl; sparingly soluble in absolute ethanol and n-butanol; and precipitating from the aqueous solution by the addition of a solution saturated with NaHCO₃;

C) an elemental analysis showing approximately 54.57 percent carbon, 6.19 percent hydrogen, 10.88 percent nitrogen, and 1.37 percent chlorine;

D) an infrared absorption spectrum in nujol with the following observable absorption maxima: 3290, 2930, and 2860 (nujol), 1765, 1635, 1510, 1455 and 1375 (nujol), 1240, 1175, 1130, 1060, 1015, 975, 840 and 815 cm⁻¹;

E) an ultraviolet absorption spectrum with the following absorption maxima:

a) in neutral methanol

234 nm ($E_{1\%}^{1\text{cm}}=206$)

268 nm ($E_{1\%}^{1\text{cm}}=114$)

b) in methanol containing 0.1N HCl

233 nm ($E_{1\%}^{1\text{cm}}=192$)

271 nm ($E_{1\%}^{1\text{cm}}=93$)

c) in methanol containing 0.1N NaOH

251 nm ($E_{1\%}^{1\text{cm}}=275$)

~300 nm (shoulder)

F) a specific rotation $[\alpha]_D^{20}$ of $+73 \pm 4^\circ$ ($c=0.49$, H₂O).

G) the following R_f values in paper chromatography on Whatman® No. 1 paper, using *B. Subtilis* ATCC 6633 as a detection organism:

Elution system (v:v:v)	R _f value
1) n-butanol saturated with Sørensen buffer pH 6.0	0.00
2) n-butanol saturated with water containing 2% of p-toluenesulfonic acid	0.00
3) n-butanol saturated with water containing 2% of ammonium hydroxide	0.00
4) Sørensen buffer, pH 6.0, saturated with n-butanol	0.05
5) n-butanol:methanol:water 4:1:2	0.48
6) ethyl acetate saturated with water	0.00
7) n-butanol:acetic acid:water 2:1:1	0.44

H) the following R_f values in the Silicagel thin-layer chromatographic systems indicated below:



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Elution system (v:v:v)

R_f value

1) aqueous 2.5% HCOONH ₄ :CH ₃ CN 65:35*	0.36
2) n-butanol:acetic acid:water 4:2:5	0.80
3) n-butanol:acetic acid:water 4:2:5	0.52
4) n-propanol:n-butanol:1N NH ₄ OH 2:3:4 (upper phase)	0.12
5) n-butanol:acetic acid:water 4:1:5	0.15

10 1,2—on silanised Silicagel 60F₂₅₄ plates

3,4,5—on Silicagel 60F₂₅₄ plates

(*Internal standards: Caffeine R_f 0.60; Cortisone R_f 0.35; Dexamethasone R_f 0.30)

15 I) an amino-acid analysis, after acidic hydrolysis in 6N hydrochloric acid at 110°C for 6 hours, which indicates the presence of at least the following amino-acids: alanine, leucine, glycine, aspartic acid, phenylalanine, ornithine, p-hydroxyphenylglycine, and hydroxy, chloro-substituted phenylglycine.

J) a carbohydrate analysis of the acid hydrolyzate after 2 hours in 2N H₂SO₄ at 100°C which indicates the presence of D-mannose;

20 K) the following characteristic reactions

Ninhydrin (3% ethanolic solution)	positive
Molish	positive
Biuret	positive
Millon	negative
25 1% FeCl ₃ —1% K ₃ Fe(CN) ₆ aqueous	green color
KMnO ₄ (acidic)	positive
H ₂ SO ₄ conc.	negative

30 and

L) a retention time (t_R) of 4' 99/100" when analyzed by reverse phase HPLC using a μ-Bondapack® C₁₈ column (3.9 mm IDx300 mm) and a mixture HCOONH₄ 0.025 M/CH₃CN 60/40 (v/v) as the mobile phase with a flow rate of 2 ml/min (Internal standards: Anthracene t_R 35' 18/100"; α-Nitronaphthalene t_R 11' 14/100"; Toluene t_R 8' 64/100", and, in the form of its hydrochloride, by

35 M) a melting point of about 250°C (with decomposition).

2. A process for producing antibiotic A/16686 factor A₂ as defined in claim 1, which comprises culturing the strain *Actinoplanes* sp. ATCC 33076 under submerged aerobic fermentation conditions, in an aqueous nutrient medium containing assimilable sources of carbon and nitrogen, and inorganic salts, until a substantial level of antibiotic activity is produced, recovering said antibiotic activity consisting of antibiotic A/16686 factor A₂ in the form of a complex with other two minor factors named antibiotic A/16686 factors A₁ and A₃ from the fermentation medium, separating antibiotic A/16686 factor A₂ from the antibiotic complex and isolating it as an essentially pure individual antibiotic compound.

3. A process as defined in claim 2 wherein the antibiotic activity is recovered at the end of the fermentation by extraction of the mycelial mass with an organic solvent selected from lower alkanols and acetone.

4. A process as in claim 3 wherein the extracting solvent is methanol.

5. A process as defined in claim 2 wherein antibiotic A/16686 factor A₂ is separated by column chromatography using an Amberlite® X-AD column and mixtures of water and acetonitrile or mixtures of aqueous ammonium formate and acetonitrile as the eluting system.

6. A process as defined in claim 2 further characterized in that all the three factors A₁, A₂, and A₃ are separately isolated.

7. A process as in claim 2 or 6 wherein separation is achieved by reverse phase HPLC using a μ-Bondapack® C₁₈ column and mixtures of aqueous ammonium formate and acetonitrile in variable ratios as the mobile phase.

8. An antibiotic substance selected from the group consisting of antibiotic A/16686 factor A₁ free base, and its non-toxic, physiologically-acceptable acid addition salts, characterized by:

A) being a white amorphous material which melts with decomposition at about 210—220°C,

B) being very soluble in water, dimethylformamide, and aqueous methanol; soluble in methanol and ethanol; but insoluble in ethyl ether, petroleum ether and benzene;

60 C) an elemental analysis showing approximately 50.31 percent carbon, 6.00 percent hydrogen, 9.92 percent nitrogen, 1.61 percent chlorine (total content), 0.90 percent chlorine ions, residue 6.07%.

D) an infrared absorption spectrum in nujol with the following observable absorption maxima: 3290, 2930 and 2860 (nujol), 1765, 1630, 1510, 1455 and 1375 (nujol), 1260, 1235, 1175, 1150, 1130, 1060, 1015, 975, 840, and 810 cm⁻¹;

65 E) an ultraviolet absorption spectrum with the following absorption maxima:

27

a) in neutral methanol:

233 nm ($E_{1\text{ cm}}^{1\%}=202$)266 nm ($E_{1\text{ cm}}^{1\%}=106$)

b) in methanol containing 0.1N HCl

232 nm ($E_{1\text{ cm}}^{1\%}=219$)270 nm ($E_{1\text{ cm}}^{1\%}=110$)

c) in methanol containing 0.1N NaOH

251 nm ($E_{1\text{ cm}}^{1\%}=264$)

~290 nm (shoulder)

F) a specific rotation, $[\alpha]_D^{20}$, of $+57 \pm 4^\circ$ ($c=0.51$, H_2O).G) the following R_f values in paper chromatography on Whatman® No. 1 paper, using *B. subtilis* ATCC 6633 as a detection system:

	Elution system (v:v:v)	R_f value
25	1) n-butanol saturated with Sørensen buffer pH 6.0	0.00
	2) n-butanol saturated with water containing 2% of p-toluenesulfonic acid	0.00
30	3) n-butanol saturated with water containing 2% of ammonium hydroxide	0.00
	4) Sørensen buffer, pH 6.0, saturated with n-butanol	0.05
	5) n-butanol:methanol:water 4:1:2	0.48
35	6) ethyl acetate saturated with water	0.00
	7) n-butanol:acetic acid:water 2:1:1	0.44

H) the following R_f values in the Silicagel thin-layer chromatographic systems indicated below

	Elution system (v:v:v)	R_f value
40	1) aqueous 2.5% $HCOONH_4$: CH_3CN 65:35*	0.40
	2) n-butanol:acetic acid:water 4:2:5	0.80
	3) n-butanol:acetic acid:water 4:2:5	0.52
45	4) n-propanol:n-butanol; 1N NH_4OH 2:3:4 (upper phase)	0.12
	5) n-butanol:acetic acid:water 4:1:5	0.15

1,2—on silanised Silicagel 60F₂₅₄ plates3,4,5—on Silicagel 60F₂₅₄ plates* (Internal standards: Caffeine R_f 0.60; Cortisone R_f 0.35; Dexamethasone R_f 0.30).

I) an amino-acid analysis, after acidic hydrolysis in 6N hydrochloric acid at 110°C for 6 hours, which indicates the presence of at least the following amino-acids: alanine, leucine, glycine, aspartic acid, phenylalanine, ornithine, p-hydroxy-phenylglycine, and hydroxy, chloro-substituted phenylglycine.

J) a carbohydrate analysis of the acid hydrolyzate after 2 hours in 2N H_2SO_4 at 100°C which indicates the presence of D-mannose.K) a retention time (t_R) of 3' 84/100" when analyzed by reverse phase HPLC using a μ -Bondapak® C₁₈ column (3.9 mm ID x 300 mm), and a mixture $HCOONH_4$ 0.025 M/ CH_3CN 60/40 (v/v) as the mobile phase with a flow rate of 2 ml/min (Internal standards: Anthracene t_R 35' 18/100"; α -Nitronaphthalene t_R 11' 14/100"; Toluene t_R 8' 64/100").9. An antibiotic substance selected from the group consisting of antibiotic A/16686 factor A₃ free base, and its non-toxic physiologically-acceptable acid addition salts, characterized, by:

A) being a white amorphous powder which melts with decomposition at about 220°C,

B) being very soluble in water, dimethylformamide and aqueous methanol; soluble in methanol and ethanol; but insoluble in ethyl ether; petroleum ether; and benzene;



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C) an elemental analysis showing approximately 48.41 percent carbon, 5.84 percent hydrogen, 9.09 percent nitrogen, 1.68 percent chlorine (total content), 1.16 percent chlorine ions; residue 9.1%,

D) an infrared absorption spectrum in nujol with the following observable absorption maxima: 3290, 2930 and 2860 (nujol), 1765, 1635, 1510, 1455 and 1375 (nujol), 1260, 1240, 1175, 1100, 1060, 1020, 975, 840, and 805 cm^{-1} ,

E) an ultraviolet absorption spectrum with the following absorption maxima:
a) in neutral methanol

234 nm ($E_{1\%}^{1\text{cm}}=147$)

267 nm ($E_{1\%}^{1\text{cm}}=74$)

b) in methanol containing 0.1N HCl

232 nm ($E_{1\%}^{1\text{cm}}=132$)

270 nm ($E_{1\%}^{1\text{cm}}=76$)

c) in methanol containing 0.1N NaOH

250 nm ($E_{1\%}^{1\text{cm}}=202$)

~295 nm (shoulder)

F) a specific rotation, $[\alpha]_D^{20}$, of $+50 \pm 4^\circ$ ($c=0.48$, HCl N/100)

G) the following R_f values in paper chromatography on Whatman No. 1 paper, using *B. subtilis* ATCC 6633 as a detection organism:

Elution system (v:v:v)	R_f value
1) n-butanol saturated with Sørensen buffer pH 6.0	0.00
2) n-butanol saturated with water containing 2% of p-toluenesulfonic acid	0.00
3) n-butanol saturated with water containing 2% of ammonium hydroxide	0.00
4) Sørensen buffer, pH 6.0, saturated with n-butanol	0.05
5) n-butanol:methanol:water 4:1:2	0.48
6) ethyl acetate saturated with water	0.00
7) n-butanol:acetic acid:water 2:1:1	0.44

H) the following R_f values in the Silicagel thin-layer chromatographic systems indicated below:

Elution system (v:v:v)	R_f values
1) aqueous 2.5% HCOONH_4 : CH_3CN 65:35*	0.32
2) n-butanol:acetic acid:water 4:2:5	0.80
3) n-butanol:acetic acid:water 4:2:5	0.52
4) n-propanol:n-butanol:1N NH_4OH 2:3:4 (upper phase)	0.12
5) n-butanol:acetic acid:water 4:1:5	0.15

1,2—on silanised Silicagel 60F₂₅₄ plates

3,4,5—on Silicagel 60F₂₅₄ plates

(*Internal standards: Caffeine R_f 0.60; Cortisone R_f 0.35; Dexamethasone R_f 0.30).

I) an amino-acid analysis, after acid hydrolysis in 6N hydrochloric acid at 110°C for 6 hours, which shows the presence of at least the following amino-acids: alanine, leucine, glycine, aspartic acid, phenylalanine, ornithine, p-hydroxyphenylglycine, and hydroxy, chloro-substituted phenylglycine;

J) a carbohydrate analysis of the acid hydrolyzate after 2 hours in 2N H_2SO_4 at 100°C which indicates the presence of D-mannose;

K) a retention time (t_R) of 6' 84/100" when analyzed by reverse phase HPLC using a μ -Bondapak® C₁₈ column (3.9 mm ID x 300 mm), and a mixture HCOONH_4 0.025 M/ CH_3CN 60/40 (v/v)



as the mobile phase with a flow rate of 2 ml/min (internal standards: Anthracene t_R 35' 18/100"; α -Nitronaphthalene t_R 11' 14/100"; Toluene 8' 64/100").

10. Antibiotic A/16686 factors A_1 , A_2 , and A_3 as defined in claim 1, 8 and 9, whenever combined, with the exclusion of antibiotic A/16686 complex as isolated according to GB—A—2045231.

Patentansprüche

1. Antibiotikum A/16686 Faktor A_2 als eine im wesentlichen reine Einzelantibiotikumverbindung und seine nicht-toxischen, physiologisch annehmbaren Säureadditionssalze, gekennzeichnet in Form der freien Base durch:

A) das Vorliegen als ein weißes, amorphes Pulver, das bei etwa 210—220°C unter Zersetzung schmilzt;

B) gute Löslichkeit in Wasser, Dimethylformamid, wässrigem Methanol und 0,1N HCl; geringe Löslichkeit in absolutem Ethanol und n-Butanol; Ausfällung aus der wässrigen Lösung bei der Zugabe einer mit NaHCO_3 gesättigten Lösung;

C) eine Elementaranalyse, die annähernd 54,57% Kohlenstoff, 6,19% Wasserstoff, 10,88% Stickstoff und 1,37% Chlor ergibt;

D) ein Infrarotabsorptionsspektrum in Nujol mit den folgenden beobachtbaren Absorptionsmaxima: 3290, 2930 und 2860 (Nujol), 1765, 1635, 1510, 1455 und 1375 (Nujol), 1240, 1175, 1130, 1060, 1015, 975, 840 und 815 cm^{-1} ;

E) ein Ultraviolettabsorptionsspektrum mit den folgenden Absorptionsmaxima:

a) In neutralem Methanol:

234 nm ($E_{1\%}^{1\text{cm}}=206$)

268 nm ($E_{1\%}^{1\text{cm}}=114$)

b) In Methanol, das 0,1N HCl enthält:

233 nm ($E_{1\%}^{1\text{cm}}=192$)

271 nm ($E_{1\%}^{1\text{cm}}=93$)

c) In Methanol, das 0,1N NaOH enthält:

251 nm ($E_{1\%}^{1\text{cm}}=275$)

~300 nm (Schulter);

F) eine spezifische Drehung $[\alpha]_D^{20}$ von $+73 \pm 4^\circ$ ($c=0,49$, H_2O);

G) die folgenden R_f -Werte bei der Papierchromatographie an Whatman® Nr. 1-Papier unter Verwendung von B. subtilis ATCC 6633 als Detektororganismus:

Eluierungssystem (Vol./Vol./Vol.)	R_f -Wert
1) n-Butanol, das mit Sörense-Puffer vom pH 6,0 gesättigt ist	0,00
2) n-Butanol, das mit 2% p-Toluolsulfonsäure enthaltendem Wasser gesättigt ist	0,00
3) n-Butanol, das mit 2% Ammoniumhydroxid enthaltendem Wasser gesättigt ist	0,00
4) Sörensen-Puffer vom pH 6,0, der mit n-Butanol gesättigt ist	0,05
5) n-Butanol:Methanol:Wasser 4:1:2	0,48
6) Ethylacetat, das mit Wasser gesättigt ist	0,00
7) n-Butanol:Essigsäure:Wasser 2:1:1	0,44

H) die folgenden R_f -Werte in den nachstehend angegebenen Silicagel-dünnschichtchromatographischen Systemen:



Eluierungssystem (Vol./Vol./Vol.)

R_f-Wert

1) Wässriges 2,5% HCOONH ₄ :CH ₃ CN (65:35)*	0,36
2) n-Butanol:Essigsäure:Wasser 4:2:5	0,80
3) n-Butanol:Essigsäure:Wasser 4:2:5	0,52
4) n-Propanol:n-Butanol:1N NH ₄ OH 2:3:4 (obere Phase)	0,12
5) n-Butanol:Essigsäure:Wasser 4:1:5	0,15

10 1,2 an silanisierten Silicagel 60 F₂₅₄-Platten

3,4,5—an Silicagel 60 F₂₅₄-Platten

(*Interne Standards: Coffein R_f 0,60; Cortison R_f 0,35; Dexamethason R_f 0,30).

15 I) eine Aminosäureanalyse nach saurer Hydrolyse in 6N Salzsäure bei 110°C während 6 Stunden, welche das Vorliegen von wenigstens folgenden Aminosäuren zeigt: Alanin, Leucin, Glycin, Asparaginsäure, Phenylalanin, Ornithin, p-Hydroxyphenylglycin und hydroxy-, chlor-substituiertes Phenylglycin;

J) eine Kohlenhydratanalyse des sauren Hydrolysates nach 2 Stunden in 2N H₂SO₄ bei 100°C, die das Vorliegen von D-Mannose zeigt;

20 K) die folgenden charakteristischen Reaktionen:

Ninhydrin (3% ethanolische Lösung)	positiv
Molish	positiv
Biuret	positiv
Millon	negativ
25 1% FeCl ₃ —1% K ₃ Fe(CN) ₆ wässrig	Grünfärbung
KMnO ₄ (sauer)	positiv
H ₂ SO ₄ konz.	negativ

und

30 L) eine Retentionszeit (t_R) von 4' 99/100" bei Analyse durch Umkehrphasen-HPLC unter Verwendung einer μ -Bondapack®-C₁₈-Säule (3,9 mm Innendurchmesser x 300 mm) und eines Gemisches HCOONH₄ 0,025 M/CH₃CN 60/40 (Vol./Vol.) als mobile Phase mit einer Fließgeschwindigkeit von 2 ml/min (interne Standards: Anthracen t_R 35' 18/100"; α -Nitronaphthalin t_R 11' 14/100"; Toluol t_R 8' 64/100"), und, in der Form seines Hydrochlorids, durch

35 M) einen Schmelzpunkt von etwa 250°C (unter Zersetzung).

2. Ein Verfahren zur Erzeugung von Antibiotikum A/16686 Faktor A₂ wie in Anspruch 1 definiert, umfassend die Züchtung des Stammes Actinoplanes sp. ATCC 33076 unter submersen aeroben Fermentationsbedingungen in einem wässrigen Nährmedium, das assimilierbare Quellen von Kohlenstoff und Stickstoff sowie anorganische Salze enthält, bis ein wesentlicher Spiegel an antibiotischer Aktivität gebildet ist, Gewinnung der erwähnten antibiotischen Aktivität, welche aus Antibiotikum A/16686 Faktor A₂ in der Form eines Komplexes mit zwei anderen geringen Faktoren, die als Antibiotikum A/16686 Faktoren A₁ und A₃ bezeichnet werden, aus dem Fermentationsmedium, Abtrennung von Antibiotikum A/16686 Faktor A₂ aus dem Antibiotikumkomplex und Isolierung desselben als einem wesentlichen reine, antibiotische Verbindung.

45 3. Ein Verfahren wie in Anspruch 2 definiert, worin die antibiotische Aktivität am Ende der Fermentation durch Extraktion der Myzelienmasse mit einem organischen Lösungsmittel, das aus niedrigen Alkanolen und Aceton ausgewählt ist, gewonnen wird.

4. Ein Verfahren wie in Anspruch 3, worin das Extraktionslösungsmittel Methanol ist.

50 5. Ein Verfahren wie in Anspruch 2 definiert, worin Antibiotikum A/16686 Faktor A₂ durch Säulenchromatographie unter Verwendung einer Amberlite® X-AD-Säule und Gemischen aus Wasser und Acetonitril oder Gemischen aus wässrigem Ammoniumformiat und Acetonitril als Eluierungssystem abgetrennt wird.

6. Ein Verfahren wie in Anspruch 2 definiert, weiterhin gekennzeichnet dadurch, daß alle drei 55 Faktoren A₁, A₂ und A₃ gesondert isoliert werden.

7. Ein Verfahren wie in Anspruch 2 oder 6, worin die Trennung durch Umkehrphasen-HPLC unter Verwendung einer μ -Bondapack® C₁₈-Säule und Gemischen aus wässrigem Ammoniumformiat und Acetonitril in variablen Verhältnissen als die mobile Phase bewirkt wird.

8. Eine antibiotische Substanz, ausgewählt aus der aus Antibiotikum A/16686 Faktor A₁ freie 60 Base, und ihren nicht-toxischen, physiologisch annehmbaren Säureadditionssalzen bestehenden Gruppe, gekennzeichnet durch:

A) das Vorliegen als ein weißes, amorphes Material, das bei etwa 210—220°C unter Zersetzung schmilzt;

65 B) gute Löslichkeit in Wasser, Dimethylformamid und wässrigem Methanol, Löslichkeit in Methanol und Ethanol; doch Unlöslichkeit in Ethylether, Petrolether und Benzol;



C) eine Elementaranalyse, die annähernd 50,31% Kohlenstoff, 6,00% Wasserstoff, 9,92% Stickstoff, 1,61% Chlor (Gesamtgehalt), 0,90% Chlorion und 6,07% Rückstand ergibt;

D) ein Infrarotabsorptionsspektrum in Nujol mit den folgenden beobachtbaren Absorptionsmaxima: 3290, 2930 und 2860 (Nujol), 1765, 1630, 1510, 1455 und 1375 (Nujol), 1260, 1235, 1175, 1150, 1130, 1060, 1015, 975, 840 und 810 cm^{-1} ;

E) ein Ultraviolettabsorptionsspektrum mit den folgenden Absorptionsmaxima:

a) In neutralem Methanol:

233 nm ($E_{1\text{ cm}}^{1\%} = 202$)

266 nm ($E_{1\text{ cm}}^{1\%} = 106$)

b) In Methanol, das 0,1N HCl enthält:

232 nm ($E_{1\text{ cm}}^{1\%} = 219$)

270 nm ($E_{1\text{ cm}}^{1\%} = 110$)

c) In Methanol, das 0,1N NaOH enthält:

251 nm ($E_{1\text{ cm}}^{1\%} = 264$)

~290 nm (Schulter)

F) eine spezifische Drehung, $[\alpha]_D^{20}$, von $+57 \pm 4^\circ$ ($c=0,51$, H_2O);

G) die folgenden R_f -Werte bei der Papierchromatographie an Whatman® Nr. 1-Papier unter Verwendung von *B. subtilis* ATCC 6633 als Detektorsystem.

	Eluierungssystem (Vol.:Vol.:Vol.)	R_f -Wert
30	1) n-Butanol, das mit Sörensen-Puffer vom pH 6,0 gesättigt ist	0,00
	2) n-Butanol, das mit 2% p-Toluolsulfonsäure enthaltendem Wasser gesättigt ist	0,00
35	3) n-Butanol, das mit 2% Ammoniumhydroxid enthaltendem Wasser gesättigt ist	0,00
	4) Sörensen-Puffer vom pH 6,0, der mit n-Butanol gesättigt ist	0,05
	5) n-Butanol:Methanol:Wasser 4:1:2	0,48
40	6) Ethylacetat, das mit Wasser gesättigt ist	0,00
	7) n-Butanol:Essigsäure:Wasser 2:1:1	0,44

H) die folgenden R_f -Werte in den nachstehend angegebenen Silicagel-dünnschichtchromatographischen Systemen:

	Eluierungssystem (Vol./Vol./Vol.)	R_f -Wert
45	1) Wässriges 2,5% $\text{HCOONH}_4 \cdot \text{CH}_3\text{CN}$ (65:35)*	0,40
	2) n-Butanol:Essigsäure:Wasser 4:2:5	0,80
50	3) n-Butanol:Essigsäure:Wasser 4:2:5	0,52
	4) n-Propanol:n-Butanol:1N NH_4OH 2:3:4 (obere Phase)	0,12
	5) n-Butanol:Essigsäure:Wasser 4:1:5	0,15

55 1,2—an silanisierten Silicagel 60 F_{254} -Platten

3,4,5—an Silicagel 60 F_{254} -Platten

(*Interne Standards: Coffein R_f 0,60; Cortison R_f 0,35; Dexamethason R_f 0,30).

I) eine Aminosäureanalyse nach saurer Hydrolyse in 6N Salzsäure bei 110°C während 6 Stunden, welche das Vorliegen von wenigstens folgenden Aminosäuren zeigt: Alanin, Leucin, Glycin, Asparaginsäure, Phenylalanin, Ornithin, p-Hydroxyphenylglycin und hydroxy-, chlor-substituiertes Phenylglycin;

J) eine Kohlenhydratanalyse des sauren Hydrolysates nach 2 Stunden in 2N H_2SO_4 bei 100°C , die das Vorliegen von D-Mannose zeigt;

65 K) eine Retentionszeit (t_R) von $3' 84/100''$ bei Analyse durch Umkehrphasen-HPLC unter Verwendung einer μ -Bondapack®- C_{18} -Säule (3,9 mm Innendurchmesser x 300 mm) und eines Gemisches



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HCOONH₄ 0,025 M/CH₃CN 60/40 (Vol./Vol.) als mobile Phase mit einer Fließgeschwindigkeit von 2 ml/min (interne Standards: Anthracen t_R 35' 18/100"; α -Nitronaphthalin t_R 11' 14/100"; Toluol t_R 8' 64/100").

9. Eine antibiotische Substanz, ausgewählt aus der aus Antibiotikum A/16686 Faktor A₃ freie Base, und ihren nichttoxischen, physiologisch annehmbaren Säureadditionssalzen bestehenden Gruppe, gekennzeichnet durch:

- A) das Vorliegen als ein weißes, amorphes Pulver, das bei etwa 220°C unter Zersetzung schmilzt;
 B) gute Löslichkeit in Wasser, Dimethylformamid und wässrigem Methanol, Löslichkeit in Methanol und Ethanol; doch Unlöslichkeit in Ethylether, Petrolether und Benzol;
 C) eine Elementaranalyse, die annähernd 48,41% Kohlenstoff, 5,84% Wasserstoff, 9,09% Stickstoff, 1,68% Chlor (Gesamtgehalt), 1,16% Chlorion und 9,1% Rückstand ergibt;
 D) ein Infrarotabsorptionsspektrum in Nujol mit den folgenden beobachtbaren Absorptionsmaxima: 3290, 2930 und 2860 (Nujol), 1765, 1635, 1510, 1455 und 1375 (Nujol), 1260, 1240, 1175, 1100, 1060, 1020, 975, 840 und 805 cm⁻¹;
 E) ein Ultraviolettabsorptionsspektrum mit den folgenden Absorptionsmaxima:

a) In neutralem Methanol

234 nm ($E_{1\%}^{1\text{cm}}=147$)

267 nm ($E_{1\%}^{1\text{cm}}=74$)

b) In Methanol, das 0,1N HCl enthält:

232 nm ($E_{1\%}^{1\text{cm}}=132$)

270 nm ($E_{1\%}^{1\text{cm}}=76$)

c) In Methanol, das 0,1N NaOH enthält:

250 nm ($E_{1\%}^{1\text{cm}}=202$)

~295 nm (Schulter)

F) eine spezifische Drehung, $[\alpha]_D^{20}$, von $+50 \pm 4^\circ$ ($c=0,48$, HCl N/100,

G) die folgenden R_f -Werte bei der Papierchromatographie an Whatman® Nr. 1-Papier unter Verwendung von B. subtilis ATCC 6633 als Detektororganismus:

	Eluierungssystem (Vol./Vol./Vol.)	R_f -Wert
40	1) n-Butanol, das mit Sörensen-Puffer vom pH 6,0 gesättigt ist	0,00
	2) n-Butanol, das mit 2% p-toluolsulfonsäure händigem Wasser gesättigt ist	0,00
45	3) n-Butanol, das mit 2% Ammoniumhydroxid enthaltendem Wasser gesättigt ist	0,00
	4) Sörensen-Puffer vom pH 6,0, der mit n-Butanol gesättigt ist	0,05
	5) n-Butanol:Methanol:Wasser 4:1:2	0,48
	6) Ethylacetat, das mit Wasser gesättigt ist	0,00
50	7) n-Butanol:Essigsäure:Wasser 2:1:1	0,44

H) die folgenden R_f -Werte in den nachstehend angegebenen Silicagel-dünnschichtchromatographischen Systemen:

	Eluierungssystem (Vol./Vol./Vol.)	R_f -Wert
55	1) Wässriges 2,5 %iges HCOONH ₄ :CH ₃ CN 65:35*	0,32
	2) n-Butanol:Essigsäure:Wasser 4:2:5	0,80
	3) n-Butanol:Essigsäure:Wasser 4:2:5	0,52
60	4) n-Propanol:n-Butanol:1N NH ₄ OH 2:3:4 (obere Phase)	0,12
	5) n-Butanol:Essigsäure:Wasser 4:1:5	0,15

1,2—an silanisierten Silicagel 60 F₂₅₄-Platten

3,4,5—an Silicagel 60 F₂₅₄-Platten

(*Interne Standards: Coffein R_f 0,60; Cortison R_f 0,35; Dexamethason R_f 0,30).



I) eine Aminosäureanalyse nach saurer Hydrolyse in 6N Salzsäure bei 110°C während 6 Stunden, welche das Vorliegen von wenigstens folgenden Aminosäuren zeigt: Alanin, Leucin, Glycin, Asparaginsäure, Phenylalanin, Ornithin, p-Hydroxyphenylglycin und hydroxy-, chlor-substituiertes Phenylglycin;

J) eine Kohlenhydratanalyse des sauren Hydrolysates nach 2 Stunden in 2N H₂SO₄ bei 100°C, die das Vorliegen von D-Mannose zeigt;

K) eine Retentionszeit (t_R) von 6' 84/100" bei Analyse durch Umkehrphasen-HPLC unter Verwendung einer μ -Bondapak®-C₁₈-Säule (3,9 mm Innendurchmesser x 300 mm) und eines Gemisches HCOONH₄ 0,025 M/CH₃CN 60/40 (Vol./Vol.) als mobile Phase mit einer Fließgeschwindigkeit von 2 ml/min (interne Standards: Anthracen t_R 35' 18/100"; α -Nitronaphthalin t_R 11' 14/100"; Toluol t_R 8' 64/100").

10. Antibiotikum A/16686 Faktoren A₁, A₂ und A₃ wie in Anspruch 1, 8 und 9 definiert, wie auch immer kombiniert, mit Ausschluß von gemäß GB—A—2045231 isoliertem Antibiotikum A/16686-Komplex.

15 Revendications

1. Facteur A₂ de l'antibiotique A/16686 en tant que composé antibiotique individuel essentiellement pur, et ses sels d'addition d'acides physiologiquement acceptables non toxiques, caractérisé dans sa forme de base libre, par le fait que:

A) il se présente sous la forme d'une poudre blanche amorphe qui fond avec décomposition à environ 210—220°C;

B) il est très soluble dans l'eau, le diméthylformamide, le méthanol aqueux et HCl 0,1 N, peu soluble dans l'éthanol absolu et le n-butanol, et il précipite à partir de la solution aqueuse par l'addition d'une solution saturée avec NaHCO₃;

C) son analyse élémentaire fournit approximativement carbone 54,57%, hydrogène 6,19%, azote 10,88% et chlore 1,37%;

D) son spectre d'absorption infrarouge en nujol fournit les maxima d'absorption observables suivants: 3290, 2930 et 2860 (nujol), 1765, 1635, 1510, 1455 et 1375 (nujol), 1240, 1175, 1130, 1060, 1015, 975, 840 et 815 cm⁻¹;

E) son spectre d'absorption ultra-violet présente les maxima d'absorption suivants:

a) en méthanol neutre:

234 nm ($E_{1\%}^{1\text{cm}}=206$)

268 nm ($E_{1\%}^{1\text{cm}}=114$)

b) en méthanol contenant HCl 0,1N

233 nm ($E_{1\%}^{1\text{cm}}=192$)

271 nm ($E_{1\%}^{1\text{cm}}=93$)

c) en méthanol contenant NaOH 0,1 N

251 nm ($E_{1\%}^{1\text{cm}}=275$)

~300 nm (épaulement);

F) son pouvoir rotatoire spécifique $[\alpha]_D^{20}$, est de $+73 \pm 4^\circ$ ($c=0,49$, H₂O);

G) ses valeurs R_f en chromatographie sur papier, en utilisant le papier Whatman No. 1, et *B. subtilis* ATCC 6633 comme organisme de détection, sont les suivantes:

	Système d'élution (en volumes)	Valeur R _f
55	1) n-butanol saturé de tampon Sørensen à pH 6,0	0,00
	2) n-butanol saturé d'eau contenant 2% d'acide p-toluènesulfonique	0,00
60	3) n-butanol saturé d'eau contenant 2% d'hydroxyde d'ammonium	0,00
	4) tampon de Sørensen, pH 6,0 saturé de n-butanol	0,05
	5) n-butanol:méthanol:eau 4:1:2	0,48
65	6) acétate d'éthyle saturé d'eau	0,00
	7) n-butanol:acide acétique:eau 2:1:1	0,44



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H) il présente les valeurs R_f suivantes sur des systèmes chromatographiques sur couche mince de gel de silice:

	Système d'élution (en volumes)	Valeur R_f
5	1) HCOONH_4 aqueux à 2,5%: CH_3CN (65:35)*	0,36
	2) n-butanol:acide acétique:eau 4:2:5	0,80
	3) n-butanol:acide acétique:eau 4:2:5	0,52
10	4) n-propanol:n-butanol: NH_4OH 1 N 2:3:4 (phase supérieure)	0,12
	5) n-butanol:acide acétique:eau 4:1:5	0,15

1,2—sur plaques de gel de silice silanisé 60F₂₅₄

3,4,5—sur plaques de gel de silice 60F₂₅₄

15 (*étalons internes: caféine R_f 0,60; cortisone R_f 0,35; dexaméthasone R_f 0,30).

I) une analyse des amino acides du facteur A_2 du A/16686 après hydrolyse acide avec de l'acide chlorhydrique 6 N à 110°C pendant 6 heures, révèle la présence d'au moins les amino acides suivants: alanine, leucine, glycine, acide aspartique, phénylalanine, ornithine, p-hydroxyphénylglycine, et phénylglycine hydroxy chloro-substituée;

20 J) une analyse des hydrates de carbone de l'hydrolysate acide après 2 heures dans H_2SO_4 2 N à 100°C indique la présence de D-mannose;

K) il présente les réactions caractéristiques suivantes:

25	Ninhydrine (solution éthanolique à 3%)	positive
	Molish	positive
	Biuret	positive
	Millon	négative
30	FeCl_3 à 1%— $\text{K}_3\text{Fe}(\text{CN})_6$ à 1% aqueux	couleur verte
	KMnO_4 (acide)	positive
	H_2SO_4 concentré	négative

et

35 L) il présente un temps de rétention (t_R) de 4' 99/100" lorsqu'on l'analyse par HPLC à phases inversées en utilisant une colonne de μ -Bondapack C_{18} (diamètre intérieur de 3,9 mm x 300 mm) et un mélange de HCOONH_4 0,025 M/ CH_3CN 60/40 (en volumes) en tant que phase mobile à un débit de 2 ml/minute (étalons internes: anthracène t_R 35' 18/100"; α -nitronaphtalène t_R 11' 14/100"; toluène t_R 8' 64/100"), et sous la forme de son chlorhydrate, par le fait qu'il présente

40 M) un point de fusion d'environ 250°C (avec décomposition).

2. Procédé pour préparer le facteur A_2 de l'antibiotique A/16686 défini comme spécifié dans la revendication 1, caractérisé par le fait qu'il consiste à cultiver la souche *Actinoplanes* sp. ATCC 33076 dans des conditions de fermentation aérobie submergée, dans un milieu nutritif aqueux contenant des sources assimilables de carbone et d'azote, et des sels minéraux, jusqu'à ce qu'un niveau substantiel d'activité antibiotique soit produit, à récupérer ladite activité antibiotique constituée par le facteur A_2 de l'antibiotique A/16686 sous la forme d'un complexe avec deux autres facteurs mineurs dénommés facteurs A_1 et A_3 de l'antibiotique A/16686 à partir du milieu de fermentation, à séparer le facteur A_2 de l'antibiotique A/16686 du complexe antibiotique et à l'isoler sous la forme d'un composé antibiotique individuel essentiellement pur.

50 3. Procédé selon la revendication 2, caractérisé par le fait que l'on récupère l'activité antibiotique à la fin de la fermentation par extraction de la masse mycéliale avec un solvant organique choisi parmi les alcanols inférieurs et l'acétone.

4. Procédé selon la revendication 3, caractérisé par le fait que le solvant d'extraction est le méthanol.

55 5. Procédé selon la revendication 2, caractérisé par le fait que l'on sépare le facteur A_2 de l'antibiotique A/16686 par chromatographie sur colonne en utilisant une colonne d'Amberlite X-AD et des mélanges d'eau et d'acétonitrile ou des mélanges de formiate d'ammonium aqueux et d'acétonitrile en tant que système éluant.

60 6. Procédé selon la revendication 2, caractérisé en outre par le fait que l'on isole séparément les trois facteurs A_1 , A_2 et A_3 .

7. Procédé selon la revendication 2 ou 6, caractérisé par le fait que l'on effectue la séparation par chromatographie en phase liquide sous haute pression (HPLC) à phases inversées en utilisant une colonne de la substance mise sur le marché sous le nom de μ -Bondapack C_{18} et des mélanges de formiate d'ammonium aqueux et d'acétonitrile en proportions variables comme phase mobile.

65 8. Substance antibiotique choisie parmi le groupe comprenant la base libre du facteur A_1 de



l'antibiotique A/16686, et ses sels d'addition d'acides physiologiquement acceptables non toxiques, caractérisée par le fait que:

- A) elle se présente comme une substance amorphe blanche qui fond avec décomposition à environ 210—220°C;
- 5 B) elle est très soluble dans l'eau, le diméthylformamide et le méthanol aqueux; soluble dans le méthanol et l'éthanol; mais insoluble dans l'éther éthylique, l'éther de pétrole et le benzène;
- C) son analyse élémentaire est approximativement la suivante: carbone 50,31%, hydrogène 6,00%, azote 9,92%, chlore (teneur totale) 1,61%, ions chlore 0,90%, résidu 6,07%;
- 10 D) son spectre d'absorption infrarouge en nujol présente les maxima d'absorption observables suivants: 3290, 2930 et 2860 (nujol), 1765, 1630, 1510, 1455 et 1375 (nujol), 1260, 1235, 1175, 1150, 1130, 1060, 1015, 975, 840 et 810 cm^{-1} ;
- E) son spectre d'absorption ultra-violet présente les maxima d'absorption suivants:

a) en méthanol neutre:

15 233 nm ($E_{1\text{ cm}}^{1\%} = 202$)

266 nm ($E_{1\text{ cm}}^{1\%} = 106$)

b) en méthanol contenant HCl 0,1 N:

20 232 nm ($E_{1\text{ cm}}^{1\%} = 219$)

270 nm ($E_{1\text{ cm}}^{1\%} = 110$)

25 c) en méthanol contenant NaOH 0,1 N

251 nm ($E_{1\text{ cm}}^{1\%} = 264$)

30 ~290 nm (épaulement);

F) elle présente un pouvoir rotatoire spécifique, $[\alpha]_D^{20}$, de $+57 \pm 4^\circ$ ($c=0,51$, H_2O);

G) elle présente les valeurs R_f suivantes en chromatographie sur du papier Whatman No. 1, en utilisant *B. subtilis* ATCC 6633 comme système de détection:

35	Système d'élution (en volumes)	Valeur R_f
	1) n-butanol saturé de tampon Sörensen à pH 6,0	0,00
	2) n-butanol saturé d'eau contenant 2% d'acide p-toluènesulfonique	0,00
40	3) n-butanol saturé d'eau contenant 2% d'hydroxyde d'ammonium	0,00
	4) tampon de Sörensen, pH 6,0 saturé de n-butanol	0,05
45	5) n-butanol:méthanol:eau: 4:1:2	0,48
	6) acétate d'éthyle saturé d'eau	0,00
	7) n-butanol:acide acétique:eau 2:1:1	0,44

50 H) elle présente les valeurs R_f suivantes dans les systèmes chromatographiques sur couche mince de gel de silice indiqués ci-après:

	Système d'élution (en volumes)	Valeur R_f
55	1) HCOONH_4 aqueux à 2,5%: CH_3CN (65:35)*	0,40
	2) n-butanol:acide acétique:eau 4:2:5	0,80
	3) n-butanol:acide acétique:eau 4:2:5	0,52
	4) n-propanol:n-butanol: NH_4OH 1 N 2:3:4 (phase supérieure)	0,12
60	5) n-butanol:acide acétique:eau 4:1:5	0,15

1,2—sur plaques de gel de silice silanisé 60F₂₅₄

3,4,5—sur plaques de gel de silice 60F₂₅₄

*(étalons internes: caféine R_f 0,60; cortisone R_f 0,35; dexaméthasone R_f 0,30).



I) une analyse des amino acides du facteur A₁ du A/16686, après hydrolyse acide en acide chlorhydrique 6 N à 110°C pendant 6 heures, indique la présence d'au moins les amino acides suivants: alanine, leucine, glycine, acide aspartique, phénylalanine, ornithine, p-hydroxyphénylglycine, et phénylglycine hydroxy, chloro-substituée;

J) une analyse des hydrates de carbone de l'hydrolysate acide après 2 heures dans H₂SO₄ 2 N à 100°C indique la présence de D-mannose;

K) elle présente un temps de rétention (t_R) de 3' 84/100" lorsqu'on l'analyse par chromatographie en phase liquide sous haute pression (HPLC) à phases inversées en utilisant une colonne de substance mise sur le marché sous le nom de μ -Bondapack C₁₈ (diamètre intérieur 3,9 mm x 300 mm), et un mélange de HCOONH₄ 0,025 M/CH₃CN 60/40 (en volumes) en tant que phase mobile à un débit de 2 ml/minute (étalons internes: anthracène t_R 35' 18/100"; α -nitronaphtalène t_R 11' 14/100"; toluène t_R 8' 64/100").

9. Substance antibiotique choisie dans le groupe comprenant la base libre du facteur A₃ de l'antibiotique A/16686, et ses sels d'addition d'acides physiologiquement acceptables non toxiques, caractérisée par le fait que:

A) elle est constituée par une poudre blanche amorphe qui fond avec décomposition à environ 220°C;

B) elle est très soluble dans l'eau, le diméthylformamide et le méthanol aqueux; soluble dans le méthanol et l'éthanol; mais insoluble dans l'éther éthylique, l'éther de pétrole et le benzène;

C) son analyse élémentaire révèle approximativement: carbone 48,41%, hydrogène 5,84%, azote 9,09%, chlore (teneur totale) 1,68%, ions chlore 1,16%; résidu 9,1%;

D) son spectre d'absorption infrarouge en nujol présente les maxima d'absorption observables suivants: 3290, 2930 et 2860 (nujol), 1765, 1635, 1510, 1455 et 1375 (nujol), 1260, 1240, 1175, 1100, 1060, 1020, 975, 840 et 805 cm⁻¹;

E) son spectre d'absorption ultra-violet présente les maxima d'absorption suivants:

a) en méthanol neutre

234 nm ($E_1^{1\%}$ cm = 147)

267 nm ($E_1^{1\%}$ cm = 74)

b) en méthanol contenant HCl 0,1 N

232 nm ($E_1^{1\%}$ cm = 132)

270 nm ($E_1^{1\%}$ cm = 76)

c) en méthanol contenant NaOH 0,1 N

250 nm ($E_1^{1\%}$ cm = 202)

~ 295 nm (épaulement);

F) elle a un pouvoir rotatoire spécifique $[\alpha]_D^{20}$, de $+50 \pm 4^\circ$ ($c=0,48$, HCl N/100);

G) elle révèle les valeurs R_f suivantes en chromatographie sur papier, sur du papier Whatman No. 1, en utilisant *B. subtilis* ATCC 6633 comme organisme de détection:

	Système d'élution (en volumes)	Valeur R_f
50	1) n-butanol saturé de tampon Sørensen à pH 6,0	0,00
	2) n-butanol saturé d'eau contenant 2% d'acide p-toluènesulfonique	0,00
55	3) n-butanol saturé d'eau contenant 2% d'hydroxyde d'ammonium	0,00
	4) tampon de Sørensen, pH 6,0, saturé de n-butanol	0,05
	5) n-butanol:méthanol:eau: 4:1:2	0,48
60	6) acétate d'éthyle saturé d'eau	0,00
	7) n-butanol:acide acétique:eau 2:1:1	0,44

H) elle présente les valeurs R_f suivantes dans les systèmes chromatographiques sur couche mince de gel de silice indiqués ci-après:



Système d'élution (en volumes)

Valeur R_f

1) HCOONH_4 aqueux à 2,5%: CH_3CN (65:35)*	0,32
2) n-butanol:acide acétique:eau 4:2:5	0,80
3) n-butanol:acide acétique:eau 4:2:5	0,52
4) n-propanol:n-butanol: NH_4OH 1 N 2:3:4 (phase supérieure)	0,12
5) n-butanol:acide acétique:eau 4:1:5	0,15

10 1,2—sur plaques de gel de silice silanisé 60F₂₅₄

3,4,5—sur plaques de gel de silice 60F₂₅₄

(*étalons internes: caféine R_f 0,60; cortisone R_f 0,35; dexaméthasone R_f 0,30).

15 I) une analyse des amino acides du facteur A_3 du A/16686 après hydrolyse acide en acide chlorhydrique 6 N à 110°C pendant 6 heures révèle la présence d'au moins les amino acides suivants: alanine, leucine, glycine, acide aspartique, phénylalanine, ornithine, p-hydroxyphénylglycine et phénylglycine hydroxy, chloro-substituée;

J) une analyse des hydrates de carbone de l'hydrolysate acide après 2 heures dans H_2SO_4 2 N à 100°C indique la présence de D-mannose;

20 K) elle présente un temps de rétention (t_R) de 6' 84/100" lorsqu'on l'analyse par chromatographie en phase liquide sous haute pression (HPLC) à phases inversées en utilisant une colonne de μ -Bondapak C_{18} (diamètre intérieur 3,9 mm x 300 mm), et un mélange de HCOONH_4 0,025 M/ CH_3CN 60/40 (en volumes) en tant que phase mobile à un débit de 2 ml/minute (étalons internes: anthracène t_R 35' 18/100"; α -nitronaphtalène t_R 11' (14/100"); toluène 8' 64/100").

25 10. Facteurs A_1 , A_2 et A_3 de l'antibiotique A/16686 tels que définis dans les revendications respectives 1, 8 et 9, dans la mesure où ils sont combinés, à l'exclusion du complexe antibiotique A/16686 tel qu'isolé selon la demande de brevet britannique publiée sous le No. 2.045.231.

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