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Isolation, structure determination and biological activity of A-16686 factors A′ 1, A′ 2 and A′ 3 glycolipodepsipeptide antibiotics

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SUMMARY

When *Actinoplanes* strain ATCC 33076, the producer of A-16686 A1, A2 and A3 complex, is fermented in a suitable medium three additional factors, designated A′1, A′2 and A′3 are produced. These were isolated and characterized, and were shown to differ from the parent components of the original complex by lacking one mannose unit. Bioconversion of A factors into A′ factors was achieved by incubation with the mycelium of *Actinoplanes* ATCC 33076. Factor A′2 has better antibacterial activity than A2 against some bacteria.

INTRODUCTION

A-16686 (also known as MDL 62 198 or ramoplanin) is a new glycolipodepsipeptide antibiotic [1–3] obtained by fermentation of *Actinoplanes* ATCC 33076. It has excellent in vitro activity against aerobic and anaerobic Gram-positive bacteria including methicillin-resistant staphylococci [4] and bacteria resistant to vancomycin, ampicillin and/or erythromycin [1,3,5]. In particular, it is very active against clinical isolates of *Propionibacterium acnes* [6,7] and clostridia [1,8].

The antibiotic was first isolated as a complex of three closely related components A1, A2 and A3 (A1, 6–12%; A2, 72–86%; A3, 8–14%) in which A2 was the most abundant [2]. Structural studies [9,10] showed that A-16686 is a cyclic peptide formed from sixteen amino acids: the C-terminal amino acid (3-chloro-4-hydroxyphenylglycine) forms a lactone bond with the hydroxyl group of a β-hydroxy-asparagine. The latter is linked through a peptide bond to an additional asparagine unit which is acylated by diunsaturated fatty acids that differentiate the three members of the complex. A D-mannosyl-D-mannose unit is linked to 4-hydroxyphenylglycine-11 (Hpg-11) by a hemiacetal bond (Fig. 1).

The knowledge of the structure of the fatty acid moieties made it possible to obtain consistently up to 90% of factor A2 by adding leucine, which is a biosynthetic precursor of the desired side chain [11], to the fermentation medium. However, during fermentation experiments we found that modification of the vegetative and fermentation medium (in particular a significant decrease of the content of glucose in the latter) led to production of an antibiotic complex different from the original one. The reverse-phase HPLC profile (Fig. 2) showed, in addition to the peaks of the known factors A1, A2 and A3, three additional peaks attributed to less hydrophilic factors designated A′1, A′2 and A′3 (Table 1).

This paper deals with the isolation of A-16686 factors A′1, A′2 and A′3, their structure elucidation, the antibacterial activity of the main factor A′2, and with the biotransformation of A factors into A′ factors by treatment with *Actinoplanes* ATCC 33076.

MATERIALS AND METHODS

General

A liquid chromatograph Hewlett-Packard 1082 with ultraviolet detector (254 nm), column Brownlee RP-18, 5 μm, 220 × 4.6 mm (i.d.) with a pre-column Brownlee

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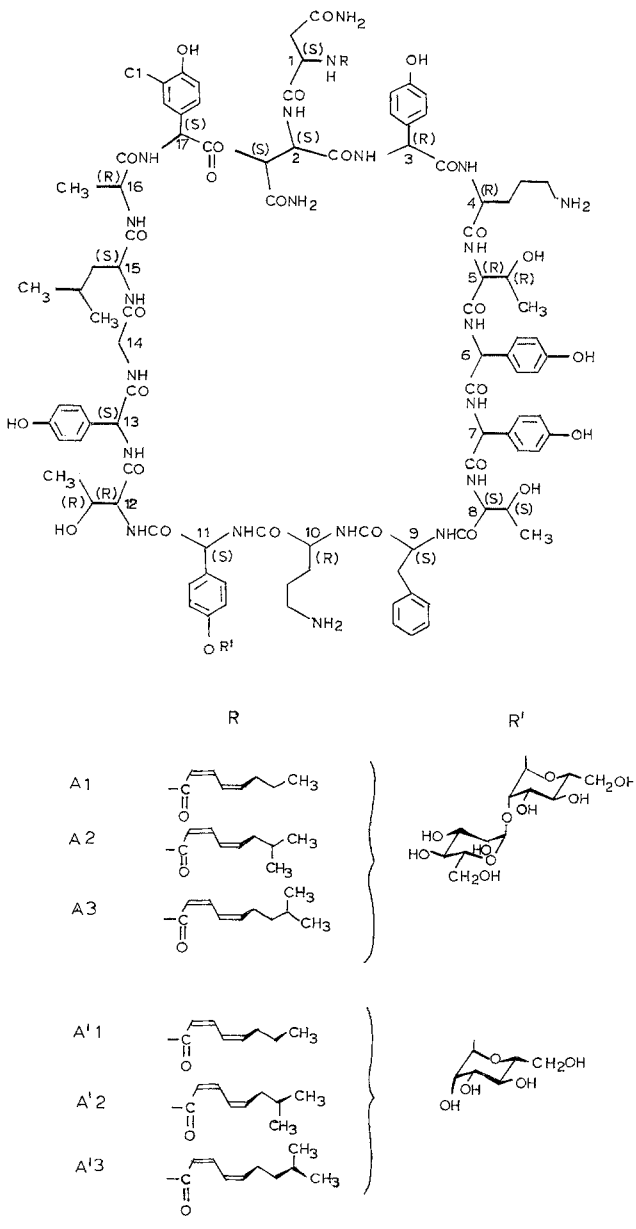

Fig. 1. Structure of A-16686A and A′ factors.

RP-18, 5 μm, 15 mm was used. HPLC conditions: mobile phase A 20 mM NaH_2PO_4/CH_3CN (9:1), phase B 20 mM NaH_2PO_4/CH_3CN (3:7); flow rate: 1.5 ml/min, injection 30 μl (2 mg/ml in CH_3CN/H_2O, 2:8). Linear gradient 45 to 75% B in 25 min.

The preparative HPLC was obtained by assembling a Waters mod 590 pump, a Waters Lambda-Max 481LC ultraviolet detector with variable wavelength set a 285 nm, and a Rheodine injector equipped with a 5 ml loop. A Lichrosorb RP-18 column (Merck), 10 μm, 250 x 50 mm (i.d.) was used. The mobile phase was 0.05 M $HCOONH_4/CH_3CN$ (64:36) with a flow rate of 30 ml/min.

FAB-MS were recorded in a positive mode with a MS9/50TC instrument using a thioglycerol/glycerol (1:1) mixture as a matrix; Xenon beam energy 6 KeV.

Strain and culture media

Actinoplanes sp. ATCC 33076 was used.

Vegetative medium (g/l): soybean meal (13), dextrose (12), dextrin (13), $CaCO_3$ (4), deionized water. Fermentation medium (g/l): soybean meal (30), glycerol (20), dextrose (4), dextrin (4), crude malt extract (20), sucrose (20), $CaCO_3$ (6), tap water. The pH of the media was adjusted to 7.3 with NaOH before sterilization.

Production of the complex containing A′1, A′2 and A′3

Fermentation. A lyophilized vial of the stock was used to inoculate two oatmeal agar slants. After incubation for 1 week at 29 °C, the mycelium of one slant was inoculated into a 500-ml Erlenmeyer flask containing 100 ml vegetative medium. The culture was incubated at 29 °C for 48 h on a shaker at 200 rpm and then transferred to a fermentor containing 4 l of vegetative medium and incubated for 48 h at 28 °C (900 rpm, 0.5 v/v per min of air). Twenty-five ml of this culture (pH 7.1) were dispensed into 50-ml ampoules. The ampoules were then frozen and stored at −74 °C.

Each of ten ampoules was inoculated into a 2-l Erlenmeyer flask containing 400 ml of vegetative medium. After 51 h of incubation at 29 °C (150 rpm) the flasks had an

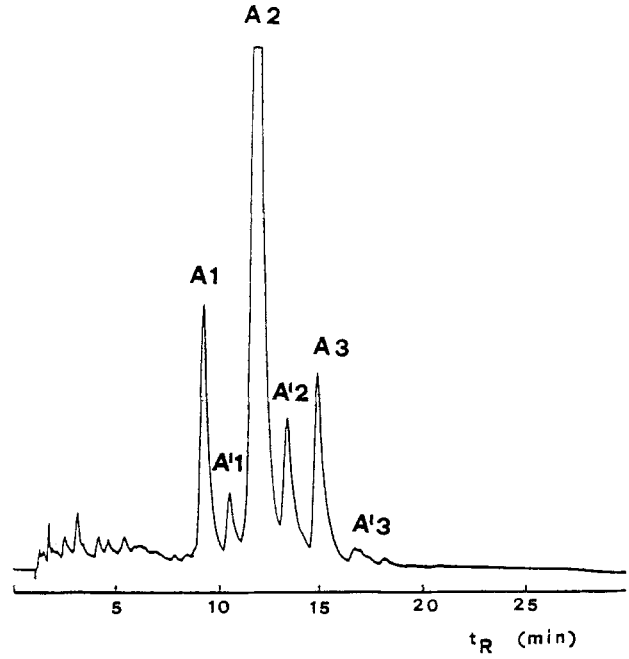

Fig. 2. HPLC profile of A-16686A and A′ factors.

average pH of 6.8. Subsequently, all the flasks were inoculated into a 10500-l fermentor containing 4000 l of vegetative medium sterilized in batch at 120 °C for 20 min.

After 68 h at 29 °C the preculture, which had a pH of 6.8, was used to inoculate two 27500-l fermentors, each containing 20000 l of fermentation medium sterilized in batch at 120 °C for 20 min. The fermentation temperature was kept constant at 29 °C and the fermentation course was monitored by HPLC.

Recovery and purification. The broth, harvested 65 h after inoculation, was cooled to 10 °C, the pH was brought to 5 with 20% HCl, and it was filtered on a rotary filter. The wet mycelium was separated and extracted three times with a total of 12500 l of acetone by stirring the suspension at pH 2 (30% HCl) and centrifuging. The water/acetone extract was divided into four portions and extracted with 12500 l of ethyl acetate. The aqueous phase was concentrated under vacuum at 18 °C (internal temperature) to 7000 l. To this solution, cooled at 4 °C, 150 l of 15% NH_4OH were slowly added until pH 7 was reached, in the presence of 210 l of CH_2Cl_2. After addition of 55 kg of filter aid the suspension was filtered and the wet cake was extracted three times with 274 l of a mixture of acetone/water (85:15) at pH 2 (20% HCl). After washing the solid with an additional 110 l of a mixture acetone/water (6:4) at pH 2, the solutions and washings (1200 l) were combined and 2600 l of acetone were added with stirring at room temperature. After standing overnight, the precipitate was centrifuged, washed with 240 l of acetone and dried at room temperature under vacuum yielding 54 kg of crude having an A-16686 titre of 28% (HPLC).

Isolation and purification of factors A'1, A'2 and A'3. A portion of the crude product (13 kg) was sludged with 80 l of 5% HCl at about 15 °C by vigorous stirring for 3 h. The suspension was centrifuged and the solid was washed with 24 l of 5% HCl. The wet cake was extracted with a mixture of 80 l of acetone and 33 l of water in the presence of decolorizing earth (Tonsil Optimum NFF) and 2 kg of charcoal (Darco G60) at 15 °C. The solid separated by centrifuging was washed with 30 l of a mixture acetone/water (6:4). The extracts were combined (140 l) and 350 l of acetone were added slowly with stirring. After standing overnight the mixture was filtered and the solid was washed with acetone/water (9:1), then with 4 l of acetone on the filter.

After drying under vacuum at room temperature 1.86 kg of the complex was obtained (as dihydrochloride). The composition (%) determined by HPLC (Fig. 2) was as follows: A1, 12.6; A'1, 3.6; A2, 49.5; A'2, 9.4; A3, 11.3; A'3, 1.8; related substances 11.8.

Twenty-seven g of the complex were submitted (300 mg for each run, dissolved in 5 ml of CH_3CN/water, 1:9) to preparative HPLC. Homogeneous fractions enriched in factors A'1, A'2, or A'3 were pooled and concentrated to dryness under vacuum. The residues were purified again by preparative HPLC as described above. The solid residues were suspended in ethanol to dissolve $HCOONH_4$ and collected by filtration. Finally, the dry solids so obtained were dissolved in 0.1 M HCl and freeze-dried to yield pure A'1 (150 mg), A'2 (600 mg), and A'3 (30 mg) as dihydrochlorides.

Bioconversion of A-16686 factors A into the corresponding factors A'. Selective demannosylation was accomplished by incubating the antibiotic with the mycelium of *Actinoplanes* ATCC 33076. This strain was grown in 100 ml of the vegetative medium described above in a 500-ml flask. After incubation for 72 h at 29 °C, 200 rpm, 40 ml of the culture was centrifuged for 10 min at 3000 rpm. The pellets were resuspended in 20 ml of distilled water containing 60 mg of A-16686 complex or factor A2 (see Tables 2 and 3). The mixtures were transferred into 500-ml sterile Erlenmeyer flasks and stirred at 200 rpm at 32 °C. The progress of transformation was monitored by the analytical HPLC method previously described. The samples were prepared as follows: 1 vol. of the mixture was brought to pH 2.0 with 20% HCl, then 1 vol. of acetone was added. The suspension was stirred for 20 min at room temperature, then centrifuged for 5 min at 3000 rpm. To 1 vol. of the supernatant, 1 vol. of ethyl acetate was added, then the water layer was separated by centrifuging for 2 min at 300 rpm and analyzed.

Biological assays. MIC was determined by macrobroth or agar dilution methods. Culture media and growth conditions: Iso-Sensitest broth (Oxoid), for staphylococci and *Enterococcus faecalis*; Todd-Hewitt broth (Difco) for streptococci; Wilkins-Chalgren agar for anaerobic bacteria; the final inoculum was about 10^4 colony-forming units/ml or spot. Incubation was 18–24 h at 37 °C (aerobes) or 48 h in $N_2/CO_2/H_2$, (8:1:1) for anaerobes.

Septicemia was induced in groups of five CD1 mice (Charles River) by intraperitoneal injection of about 10^5 cells of *Streptococcus pyogenes* C203. Mice were treated once, s.c. immediately after infection. The ED_{50} was calculated by the Spearman-Kaerber method [12] from the percentage of mice surviving to day 7 at each dose.

LD_{50} was determined in groups of five CD1 mice (Charles River) of both sexes weighing 18–22 g. It was calculated by the probit analysis.

RESULTS AND DISCUSSION

Structural determination

The structures of factors A'1, A'2, and A'3 (complex A') were elucidated by comparing the analytical data (Table 1) with those obtained from the corresponding factors A1, A2, and A3 (complex A) [9,10].

TABLE 1

Properties of A′ factors in comparison with A,A′ complex

	HPLC retention time (min)[a]	Elemental analysis (%)[b]					Formula	MW	MW[c]	$[M+H^+]$ cluster peaks[d] *m/z*
		C	H	Cl	N	ashes				
Factor A′1	10.49 (9.20)	54.2	5.8	3.9	11.8	0.9	$C_{112}H_{142}ClN_{21}O_{35}$	2377.926	2375	2377,78,79,80 (93,100,97,90)
Factor A′2	13.26 (11.64)	55.2	5.4	4.2	12.6	1.3	$C_{113}H_{144}ClN_{21}O_{35}$	2391.953	2389	2391,92,93,94 (65,100,85,75)
Factor A′3	16.62 (14.76)	55.8	6.2	4.0	11.4	0.7	$C_{114}H_{146}ClN_{21}O_{35}$	2405.980	2403	–[e]
A,A′ complex[f]	–	53.7	6.0	4.6[g]	11.0	1.0	–	–	–	–

[a] The retention times of the corresponding A factors are shown in brackets.
[b] Determined on samples previously dried at 140 °C in inert atmosphere. The inorganic residue was determined after heating the samples at 900 °C in oxygen atmosphere.
[c] Low isotope composition.
[d] The relative abundances are shown in brackets.
[e] Peaks not sufficiently resolved.
[f] The product contains also; H_2O (K. Fisher), 6.2%; Me_2CO (gas chromatography), 0.7%.
[g] Cl^- found (%): 2.92.

The FAB-MS spectra gave isotopic clusters of the cationized molecular ions indicating molecular masses of 2375, 2389 and 2403 daltons (lowest isotope compositions) consistent with the lack of the mannose, and fragment ions corresponding to the loss of 163 units, attributed to one mannose unit, from the respective MH^+ ions. The molecular formulas shown in Table 1 were derived. They clearly differ from factors A1, A2 and A3, which showed loss of two mannose units [9].

The infrared spectra (nujol) showed practically the same bands as for the A components, in particular the presence of absorption maxima at 3500–3100 (ν NH and νOH), 1760 (ν C=O, lactone), 1630 (νC=O, amide I), 1510 (δ NH, amide II), 1225 (ν C–O, lactone), 1065–980 (ν C–O, sugar), 840 and 815 cm^{-1} (γCH aromatic). The ultraviolet spectra in water exhibited absorption maxima at 232 and 270 nm. Amino acid composition and chiralities of A′1, A′2 and A′3 were done as described [9], and were found to correspond to those of the respective A factors.

All this evidence strongly supported the hypothesis that factors A′1, A′2 and A′3 had the same core structure of the factors A1, A2 and A3 and that the only difference was the lack of one mannose unit. This was eventually confirmed by homonuclear two-dimensional NMR studies (H_2O/DMSO (4:1), pH 4.6, 40 °C, internal standard TMS, δ0.00 ppm) that gave chemical shift values corresponding to those of A1, A2 and A3 for the amino acid skeleton and fatty acid residues [10], while the signals corresponding to the mannose unit at δ5.40 (anomeric proton), δ4.01, 3.91–3.71, 3.60–3.51 (other protons) accounted for one mannose unit only. NOESY measurements showed connectivities between the *ortho* and *meta* protons of Hpg-11 and the anomeric proton of the dimannose unit at δ5.40, thus establishing the position of the remaining mannose moiety.

Therefore, on the basis of the above findings it is evident that each A′ factor differs from the corresponding A factor in lacking one mannose unit (Fig. 1). An antibiotic designated UK-71903 or ramoplanose, that differs from A-16686 factor A2 in having a trimannosyl branched chain and a *cis*-transoid-*trans* N-terminal fatty acid has been recently described [13].

TABLE 4

Conversion of A-16686 factors A into A-16686 factors A′ by *Actinoplanes* ATCC 33076

Time (h)	Composition[a] (%)						Yield[b] (%)
	A1	A′1	A2	A′2	A3	A′3	
0	14.3	4.1	56.1	10.7	12.8	2.0	100
2	12.9	5.7	52.9	13.4	12.8	2.3	95
24	9.2	9.4	41.1	24.5	11.9	3.9	73
48	8.9	10.3	36.9	27.7	11.7	4.5	49
72	8.6	10.7	33.6	30.5	11.3	5.3	41
96	6.4	11.2	32.1	33.4	10.8	5.1	39

[a] Percentage distribution of the areas of the peaks of the complex determined by HPLC.
[b] Calculated on the sum of the areas of the peaks. Time 0 assumed as 100%.

TABLE 3

Conversion of A-16686 factor A2 into A-16686 factor A'2 by *Actinoplanes* ATCC 33076

Time (h)	Composition[a] (%)		Yield[a] (%)
	A2	A'2	
0	90.0	10.0	100
24	68.0	32.0	55
48	58.1	41.9	46
72	53.3	46.6	31
96	50.0	50.0	28

[a] See notes to Table 2.

Bioconversion of A-16686 factors A into the corresponding factors A'

As shown in Table 2, A factors contained in the original complex were converted by *Actinoplanes* ATCC 33076 into the A' factors in an approximate 50% yield in 96 h, although about 60% of the product was lost. Also, when the transformation was carried out on material highly enriched in A2 factor, factor A'2 was formed in a consistent yield (Table 3). HPLC analyses did not reveal any derivative opened at the lactone bond [9] or the presence of the aglycones [14] by comparison with authentic samples.

TABLE 4

In vitro antimicrobial activity

Test organism	Strain	MIC (μg/ml)	
		factor A'2	A-16686[a]
Staphylococcus aureus	L165	1	1
S. epidermidis	ATCC 12228	0.25	0.5
S. haemolyticus	L602[b]	1	0.5
Streptococcus pyogenes	C203	0.008	0.016
S. pneumoniae	UC41	0.008	0.032
S. mitis	L796[b]	0.032	0.125
Enterococcus faecalis	ATCC 7080	0.5	0.5
Clostridium perfringens	ISS 30543	2	1
C. difficile	ATCC 9689	1	1
Propionibacterium acnes	ATCC 6919	0.5	0.25
P. acnes	ATCC 6922	0.125	0.125
P. acnes	L1557[b]	0.125	0.125
P. acnes	L1559[b]	0.125	0.125
P. acnes	L1563[b]	0.125	0.125
P. acnes	L1565[b]	0.125	0.125

[a] Composition by HPLC, % of areas: A2 82.8; minor factors A1 1.8, A'2 9.1, A3 1.6; related substances 4.7.

[b] Clinical isolates.

Antibiotics belonging to different classes such as penicillin [15], lipopeptides [16,17], glycopeptides [18], macrolides [19], are deacylated by microorganisms of the family Actinoplanaceae, particularly by strains of the genus *Actinoplanes*. Only one example is described of desmethylation of a macrolide compound by using *Actinoplanes* cultures [20]. On the other hand, although some cases of biodemannosylation of glycopeptide antibiotics are reported [21], no example of use of *Actinoplanes* is given.

We have shown that *Actinoplanes* ATCC 33076 is very effective in bio-transforming a glycolipopeptide by selective demannosylation in the presence of acylamido functions.

Biological activity

The MICs of factor A'2 were compared with those of an A-16686 sample containing 82.8% of factor A2 (Table 4). Factor A'2 appeared more active than A-16686 against the *S. pneumoniae* and *S. mitis* strains; the two products showed the same activity against clinical isolates of *Propionibacterium acnes*.

Factor A'2 was as effective as A-16686 in curing *S. pyogenes* experimental septicemia in the mouse (ED_{50} 0.11 and 0.08 mg/kg, respectively), and the i.v. acute toxicities in mice were similar (LD_{50} 103 and 110 mg/kg, respectively). The activities of factors A'1 and A'3 were not determined; previous data showed that the activities of A1, A2 and A3 do not differ substantially [3].

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