ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY OF PAMAMYCIN-607, AN AERIAL MYCELIUM-INDUCING SUBSTANCE FROM *STREPTOMYCES ALBONIGER*

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Pamamycin-607, which showed aerial mycelium-inducing activity, has been isolated from *Streptomyces alboniger* IFO 12738. At 0.1 μg/disc it induces aerial mycelia in the aerial mycelium-negative strain of *S. alboniger* but inhibits growth of the substrate mycelia at 10 μg/disc. It also acts as an antibiotic against some fungi and bacteria. When $KMnO_4$ was partitioned with pamamycin-607 between benzene and water, MnO_4^- but no K^+ was transferred from the water to the benzene layer; pamamycin-607 was thus shown to be a novel anion-transfer antibiotic.

Actinomycetes, which produce a number of useful antibiotics, have a life cycle that is complex when compared with the various procaryotic microorganisms. This life cycle starts with germination of the spores, which then extend filamentous vegetative mycelia (substrate mycelia) on (and into) the agar medium. Subsequently, the hyphae differentiate and grow in the air as aerial mycelia. Spores are produced on each aerial mycelium in most actinomycete species.[1] Recently, the relation between cytodifferentiation and secondary metabolism in actinomycetes has attracted much attention, a positive correlation having been reported in several species. Both the ability to produce aerial mycelia and an antibiotic is apt to be lost simultaneously when species undergo mutation or are cultured at a high temperature.[2] A spontaneous mutant of *Streptomyces lactamdurans* that lacked aerial mycelia failed to produce the antibiotic cephamycin C.[3] The A-factor, first isolated from the normal strain of *Streptomyces griseus* by Khokhlov *et al.*, restored both the ability to produce streptomycin and form spores in a *S. griseus* strain deficient in these abilities.[4]

We are interested in the endogenous regulating substances present during the formation of aerial mycelia in actinomycetes and have expanded our research on the isolation and chemical characterization of these bioactive metabolites. Consequently, we have isolated 2-methyl-4-amino-5-hydroxymethylpyrimidine (toxopyrimidine) from a *Streptoverticillium* species.[5] In the presence of inosine (or other purine bases) it induced formation of aerial mycelia in a spontaneous bald (aerial mycelium-negative) strain of the same species. We subsequently found that $Ca(OAc)_2$ was the active substance in *Streptomyces ambofaciens*. Ca^{2+} induced or accelerated aerial mycelium formation in a wide range of species, and the formation of aerial mycelia naturally or by the addition of $CaCl_2$ was inhibited by ethylene glycol bis(2-aminoethylether) tetraacetic acid (EGTA), a Ca^{2+} specific chelating reagent. We, in fact, have evidence that Ca^{2+} has an important function in regulating the formation of aerial mycelia in many species of actinomycetes (M. Natsume *et al.*; manuscript in preparation).

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Fig. 1.   Structure of pamamycin-607.
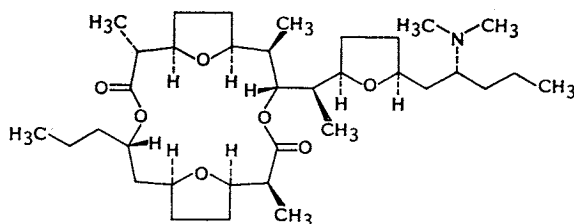
We found that a normal strain of *Streptomyces alboniger* IFO 12738 that forms abundant aerial mycelia produced aerial mycelium-inducing substances when cultured on an inorganic salts - starch (ISS) agar.   These active substances were extracted from the culture material with methanol. McCann and Pogell (1979) had reported earlier that *S. alboniger* produced an aerial mycelium-inducing substance named pamamycin.   They obtained a mixture of four pamamycin homologues and postulated the molecular formula, $C_{36}H_{63}NO_7$, for the major component (MW 621) of the pamamycin complex.   Its structure, however, could not be elucidated.[6]

We have now isolated a new pamamycin homologue, MW 607, in pure form from *S. alboniger* IFO 12738.   The isolated compound, named pamamycin-607, at 0.1 μg/paper disc, induced aerial mycelium formation in an aerial mycelium-negative spontaneous mutant of the producing strain.   It also showed antimicrobial activity against some phytopathogenic fungi and Gram-positive bacteria. The structure of pamamycin-607 and its relative stereochemistry (Fig. 1) have been determined by 2D ^{1}H-^{13}C and ^{1}H-^{1}H correlation NMR and nuclear Overhauser effect (NOE) difference spectroscopy, as already reported elsewhere.[7]

We describe here the isolation, physico-chemical properties, biological activities and anion transfer ability of pamamycin-607.

Materials and Methods

General
The UV spectrum was recorded with a Jasco Uvidec 505 spectrometer and the IR spectrum with a Jasco A-3 IR spectrometer.   Mass spectra were measured with Jeol JMS D-100 or JMS DX-300 apparatus and the optical rotation with a Jasco DIP-4 polarimeter.   The ^{1}H and ^{13}C NMR spectra were recorded with Jeol JNM FX-200 or JNM GX-500 spectrometer.

Microorganisms
S. alboniger IFO 12738 was used for the fermentation that produced pamamycin-607.   The aerial mycelium-negative mutant (AM⁻-1) used in the bioassay was obtained by spontaneous mutation of *S. alboniger* IFO 12738 that had been cultured for 10 days at 30°C on a slant composed of ISS agar medium (ISP-4).[8]   Spores produced by this culture were scraped from the agar slant and suspended in distilled water containing 0.1% Tween 80 and glass beads (diameter 1.0~1.5 mm).   By treatment with a Vortex mixer all the mycelia ruptured and all the spores were separated.   The spore suspension was filtered through Toyo No. 5B filter paper.   The filtrate containing unicellular spores was diluted 1,000-fold with distilled water containing 0.1% Tween 80.   The diluted spore suspension then was plated on Hickey and Tresner's (HT) agar medium[9] in petri dishes.   After 3 days of incubation at 30°C, the aerial mycelium-negative colonies that had formed were picked up and transferred to new HT agar slants which were cultured for 7 days at 30°C.   The 26 strains that remained aerial mycelium-negative were replated on HT agar medium in petri dishes, and their ability to regain aerial mycelium formation was examined as follows: The normal strain of *S. alboniger* was cultured on ISS agar medium

for 7 days at 30°C. Paper discs (8 mm diameter) were impregnated with the MeOH extract of the cultured material and, after the solvent had evaporated, each disc was placed on an agar plate that had been inoculated with an aerial mycelium-negative strain. After incubation of these plates for 4 days at 30°C, only one strain (AM⁻-1) showed a restored ability to form aerial mycelia around the paper disc (0.008 % of the total colonies). This was used as the bioassay strain for isolating the aerial mycelium-inducing substances.

The test microorganisms used for antifungal and antibacterial activity were obtained from ATCC, IFO and Hokko Chemical Industry Co., Ltd.

Fermentation

A seed culture was grown by transferring a loopful of *S. alboniger* IFO 12738 from an ISS agar slant to a 500-ml shaking flask that contained 125 ml of ISS medium. The inoculated flask was shaken on a reciprocal shaker (150 rpm) for 7 days at 30°C, after which 1-ml portions of its broth were used to inoculate shaking flasks that each contained 125 ml of the same medium. These inoculated flasks were cultured with shaking under conditions similar to those used for the seed culture.

Bioassay for Aerial Mycelium-inducing Activity

Aerial mycelium-inducing activity was bioassayed by the paper-disc method using the aerial mycelium-negative strain (AM⁻-1) of *S. alboniger*. A plate (9 cm diameter) containing 10 ml of HT agar medium was smeared with the aerial mycelium-negative strain. A test sample was impregnated in a paper disc (8 mm diameter) and, after the solvent had evaporated, the disc was placed on the inoculated plate. After 2 days incubation at 30°C, the diameter of the zone of aerial mycelium was measured.

Isolation

The cultured broth (200 liters) was separated into a filtrate and mycelia by filtration. The filtrate was treated three times with EtOAc. The mycelia were treated with MeOH and, after being concentrated to an aqueous residue, the MeOH extract was treated with EtOAc. The EtOAc extract obtained was combined with the extract from the filtrate. The combined extracts were concentrated to 2 liters and washed with satd aq NaHCO₃ to give neutral (and basic) fraction which was evaporated to dryness. The neutral substances obtained (9.1 g) were applied to a column of Silica gel BW-820MH (300 g, Fuji Davison Chemicals Co.). This column was washed with EtOAc (1.8 liters) and the fraction containing the activity was eluted with 1 % diethylamine in EtOAc (1.8 liters). The active eluate was evaporated to dryness *in vacuo*, and its residue (384 mg) was rechromatographed on Silica gel BW-200 (30 g, Fuji Davison Chemicals Co.). The column was eluted in steps with 0, 5, 10 and 20 % EtOAc in *n*-hexane, 1 % diethylamine being added to every solvent mixture. All activity was recovered in the fraction eluted with *n*-hexane - EtOAc - diethylamine (95 : 5 : 1). This active fraction (240 mg) contained most of the pamamycin homologues, but no other substances. The pamamycin homologues were separated from each other by chromatographing the active fraction on a column of TLC Kieselgel 60H (50 g, average particle size 15 μm, E. Merck Co.) using a 5~15 % stepped gradient of EtOAc in *n*-hexane, with 3~5 % diisopropylamine was added at each step. The eluates were collected in 10-ml aliquots and analyzed on an HPTLC plate NH₂ (E. Merck Co.) with the solvent system *n*-hexane - MeOH - *n*-butylamine (10 : 0.15 : 0.25). Fraction numbers 75~108, which mainly contained a pamamycin homologue with the MW of 607, were combined and rechromatographed on TLC Kieselgel 60H. Pamamycin-607 finally was purified twice by HPLC on Zorbax NH₂ (Du Pont Co.) with *n*-heptane - MeOH - *n*-butylamine (10 : 0.05 : 0.05).
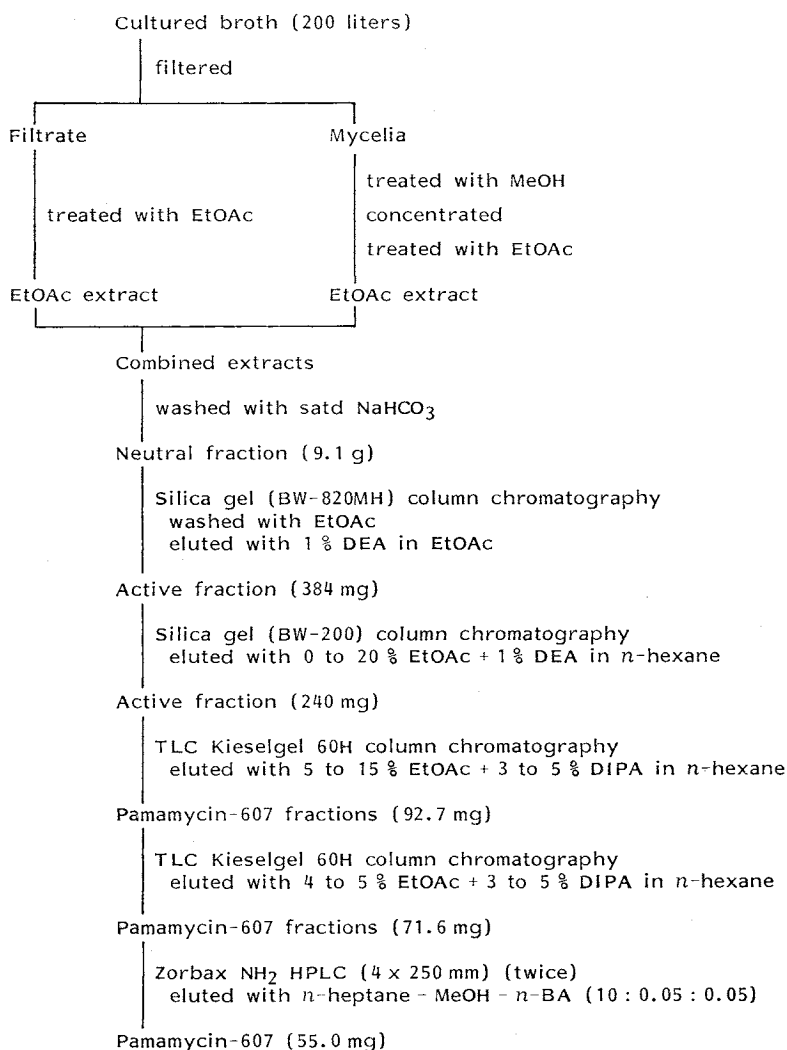
Antimicrobial Activity

The antimicrobial activity of pamamycin-607 was determined by serial dilution with nutrient - agar medium for the bacteria tested and with potato - sucrose agar medium for the fungi. The MIC is expressed as the number of μg/ml after 24 hours of incubation at 37°C for bacteria and after 48 hours at 27°C for fungi.

Ion Transfer Ability

The ion transfer ability of pamamycin-607 was examined by a two-phase partition experiment[10]

in two ways.   A): A solution of pamamycin-607 (0.1 mM, 4.0 ml) in toluene - BuOH (7 : 3) was mixed with an aqueous solution (5.0 mM, 2.0 ml) of KCl, NaCl or CaCl$_2$ in a centrifuge tube.   After shaking on a reciprocal shaker at 150 rpm at 28°C for 24 hours, the tube was centrifuged at 2,000 rpm for 20 minutes to separate the phases.   The amount of K$^+$, Na$^+$ and Ca^{2+} that had transferred to the organic phase was measured by atomic absorption spectrometry.   For measuring K$^+$ and Ca^{2+}, the organic phase (3.0 ml) was evaporated to dryness *in vacuo* and the residue was ashed with H$_2$SO$_4$ (0.2 ml) at 200°C in Pyrex tube.   The resultant ash was then dissolved in 0.5 N HCl (2.0 ml), and K$^+$ or Ca^{2+} in the HCl solution was quantified with a Hitachi 170-50A atomic absorption spectrophotometer.   For Na$^+$, the organic phase was evaporated to dryness, the residue was, without being ashed with H$_2$SO$_4$, dissolved in 0.5 N HCl, and the amount of Na$^+$ was analyzed as described above. B): A benzene solution of pamamycin-607 (0.1 mM, 2.0 ml) was mixed with a solution of KMnO$_4$ (20 mM, 1.0 ml) dissolved in distilled water, 0.01 N HCl or 0.01 N KOH.   The mixture was then put in a Pyrex tube with Teflon-lined screw cap, and shaken on a reciprocal shaker under the above conditions for 2 hours.   The tube was centrifuged at 1,500 rpm for 20 minutes, and the amount of MnO$_4$$^-$

Fig. 2.   Isolation procedure for pamamycin-607.


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Cultured broth (200 liters)
        |
        | filtered
        |
   -----------------------------------
   |                                 |
Filtrate                          Mycelia
   |                                 |
   |                                 | treated with MeOH
   | treated with EtOAc              | concentrated
   |                                 | treated with EtOAc
   |                                 |
EtOAc extract                    EtOAc extract
   |                                 |
   -----------------------------------
        |
   Combined extracts
        |
        | washed with satd NaHCO3
        |
   Neutral fraction (9.1 g)
        |
        | Silica gel (BW-820MH) column chromatography
        |  washed with EtOAc
        |  eluted with 1 % DEA in EtOAc
        |
   Active fraction (384 mg)
        |
        | Silica gel (BW-200) column chromatography
        |  eluted with 0 to 20 % EtOAc + 1 % DEA in n-hexane
        |
   Active fraction (240 mg)
        |
        | TLC Kieselgel 60H column chromatography
        |  eluted with 5 to 15 % EtOAc + 3 to 5 % DIPA in n-hexane
        |
   Pamamycin-607 fractions (92.7 mg)
        |
        | TLC Kieselgel 60H column chromatography
        |  eluted with 4 to 5 % EtOAc + 3 to 5 % DIPA in n-hexane
        |
   Pamamycin-607 fractions (71.6 mg)
        |
        | Zorbax NH2 HPLC (4 x 250 mm) (twice)
        |  eluted with n-heptane - MeOH - n-BA (10 : 0.05 : 0.05)
        |
   Pamamycin-607 (55.0 mg)
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DEA: Diethylamine, DIPA: diisopropylamine, *n*-BA: *n*-butylamine.

transferred to the benzene phase was determined by measuring the UV absorbance at 528 nm. The K^+ concentration in the benzene phase was determined by evaporating the benzene (1.0 ml) to dryness in a Pyrex tube, and the residue was, after dissolving in 10% H_2SO_4 (1.0 ml) with sonicating for 5 minutes, analyzed by atomic absorption spectrometry. Valinomycin and tetra-*n*-butylammonium chloride were partitioned between benzene and water containing $KMnO_4$, respectively, and the amounts of MnO_4^- and K^+ in the benzene phase were quantified under experimental conditions similar to those used for pamamycin-607.

Results

Isolation

The isolation procedure for pamamycin-607 is outlined in Fig. 2. Fractions of the column chromatography on TLC Kieselgel 60H were analyzed by HPTLC plate NH_2 and also by MS to determine their MWs. Results shown in Fig. 3, indicate that there are at least 8 pamamycin homologues with MWs ranging from 593 to 691, each separated by a difference of 14 mass units. Fig. 4 shows the chromatogram of the first HPLC on Zorbax NH_2. The shaded portion of the peak was collected and rechromatographed on the same column. Thus, 55 mg of pure pamamycin-607 was obtained as a single peak.

Physico-chemical Properties

The physico-chemical properties of pamamycin-607 were already reported elsewhere.[7] Its mass and ^{1}H NMR spectra are shown in Figs. 5 and 6, respectively. Its molecular formula was $C_{35}H_{61}NO_7$ by high-resolution electron impact (HREI)-MS and ^{13}C NMR spectrometry. Pamamycin-607 is soluble in acetone, acetonitrile, methanol, pyridine, benzene and chloroform but is almost insoluble in water.

Fig. 3. HPTLC chromatogram of pamamycin homologues fractionated by TLC Kieselgel 60H column chromatography.
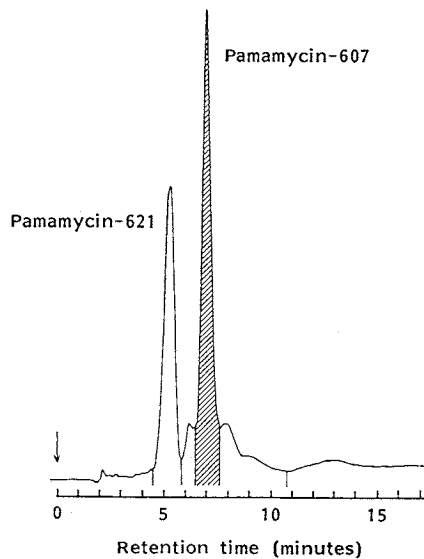

Adsorbent: HPTLC plate NH_2 F_{254}. Developing solvent: *n*-hexane - MeOH - *n*-butylamine (10:0.15: 0.25). Detection: vanillin - sulfuric acid. Numbers give the MW of the pamamycin homologues.

The structure of pamamycin-607 shown in Fig. 1 is based on spectral analysis by 2D ^{1}H-^{13}C, ^{1}H-^{1}H correlation NMR and NOE difference spectroscopies.[7] It has a novel sixteen-membered macrodiolide ring with a dimethylamino group-bearing side chain.

Aerial Mycelium-inducing Activity

The aerial mycelium-inducing activity of pamamycin-607 was assayed with an aerial mycelium-negative strain (AM$^-$-1) of *S. alboniger* IFO 12738 that we collected as a spontaneous bald strain. Its inducing activity is shown in Fig. 7 and Table 1. At a dose of 0.1 μg/disc, pamamycin-607 produced a definite zone of aerial mycelia, its activity increasing as the amount of the compound increased (Table 1). Above 10 μg/disc, pamamycin-607 inhibited vegetative growth rather than induced the formation of aerial mycelia. Pamamycin-607 stimulated

Fig. 4. HPLC chromatogram of pamamycin-607 fractions.

Column: Zorbax NH$_2$ (4×250 mm). Mobile phase: *n*-heptane - MeOH - *n*-butylamine (10:0.05:0.05). Flow rate: 1 ml/minute. Detection: UV 230 nm.
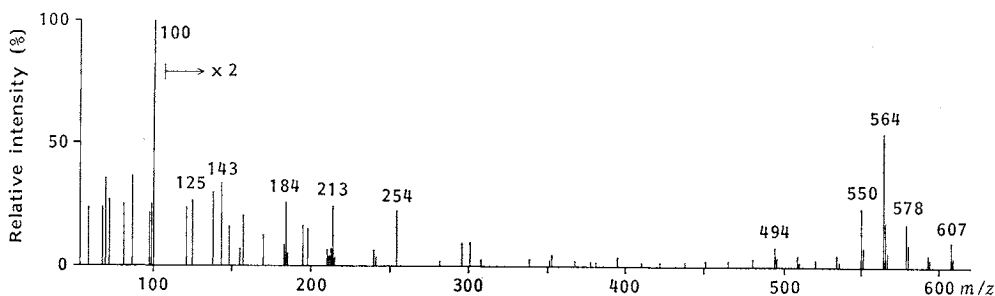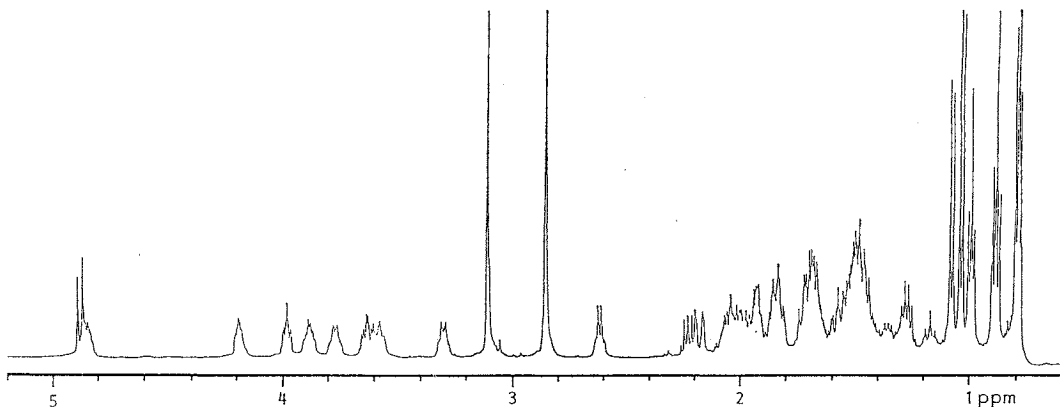
Fig. 5. Mass spectrum of pamamycin-607.

Fig. 6. ^{1}H NMR spectrum of pamamycin-607·CF$_3$COOD (500 MHz in CDCl$_3$).

aerial mycelium formation in the parent strain, *S. alboniger* IFO 12738 (data not shown).

Antimicrobial Activity

The antimicrobial spectrum of pamamycin-607 is shown in Table 2. Pamamycin-607 showed strong activity against *Cochliobolus miyabeanus* and *Diaporthe citri*, and moderate activity against *Bacillus subtilis* and *Bacillus cereus*.

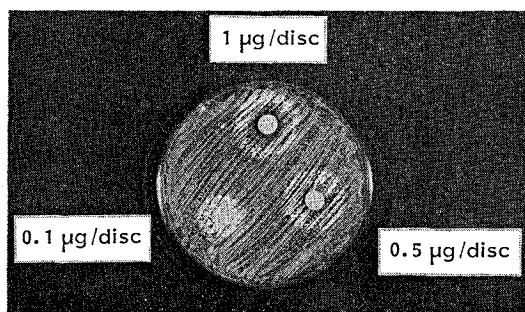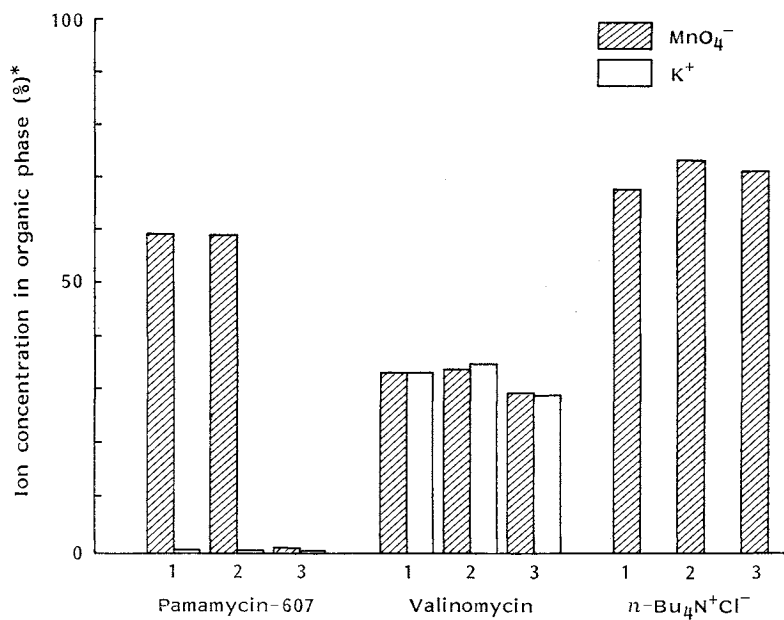Fig. 7. Aerial mycelium-inducing activity of pamamycin-607.

Table 1. Effect of pamamycin-607 against the aerial mycelium-negative mutant (AM⁻-1) of *Streptomyces alboniger* IFO 12738.

Dose (μg/disc)	Aerial mycelium induction* (mm)	Substrate mycelium inhibition* (mm)
50	45	31
10	37	19
5	31	0
1	21	0
0.5	18	0
0.1	12	0
0.05	±	0

* Activities are given as the diameters of the active zones (see Fig. 7).

Fig. 8. The MnO_4^- and K^+ transfer ability of pamamycin-607, valinomycin and tetra-*n*-butylammonium chloride.

1: 0.01 N HCl, 2: H_2O, 3: 0.01 N KOH.

$$*\% = \frac{\mu mol\ of\ MnO_4^-\ or\ K^+\ transferred\ to\ benzene\ layer}{0.2\ \mu mol\ of\ a\ phase\ transfer\ compound\ added\ in\ benzene\ layer} \times 100$$

Ion Transfer Ability

In two-phase partition systems between toluene - BuOH (7:3) and aqueous solutions of KCl, NaCl or CaCl$_2$, addition of pamamycin-607 did not result in transfer of K$^+$, Na$^+$ or Ca^{2+} from the aqueous phase to the organic phase. In contrast, when KMnO$_4$ was partitioned between benzene containing pamamycin-607 and water, only MnO$_4^-$ and no K$^+$ was transferred from the water to the benzene layer under neutral and acidic but not under alkaline conditions (Fig. 8). Valinomycin, which is a cation ionophore, transferred almost similar amounts of MnO$_4^-$ and K$^+$ from the aqueous to the benzene phase. Tetra-n-butylammonium chloride, a phase transfer catalyst, transferred only MnO$_4^-$ and not K$^+$ under acidic, neutral and alkaline conditions. The characteristic difference between these known phase transfer compounds and pamamycin-607 is that the ion transfer abilities of the former two compounds are independent of the pH. Pamamycin-607 could transfer the anion only when the dimethylamino group of its side chain was in the ammonium salt form.

Table 2. Antimicrobial spectrum of pamamycin-607.

Organism	MIC (μg/ml)
Alternaria kikuchiana	25
A. mali	12.5
Aspergillus niger ATCC 6275	25
Candida albicans 3147	25
Cochliobolus miyabeanus	1.56
Diaporthe citri IFO 6443	1.56
Fusarium oxysporum f. sp. FO-1	12.5
Gibberella fujikuroi	6.25
Rhizoctonia solani	12.5
Sclerotinia sclerotiorum	12.5
Bacillus subtilis ATCC 6633	3.13
B. cereus	3.13
Erwinia carotovora subsp. *carotovora*	6.25
Pseudomonas fluorescens	100
P. syringae pv. *lachrymans*	25
Xanthomonas campestris pv. *campestris*	6.25

Discussion

During the purification procedure, pamamycins were tightly adsorbed on a silica gel column in the absence of an amine, but could be eluted by the addition of an amine to the eluting solvent. This property was used to separate pamamycin homologues from the other metabolites. The addition of a small amount of amine to the chromatographic solvent system also made possible the mutual separation of pamamycin homologues.

The pamamycin complex consists of eight or more homologous compounds ranging in MW from 593 to 691. We have isolated one component with a MW of 607 and named it pamamycin-607.

In the actinomycetes, several butyrolactone-type compounds, such as A-factor, have been isolated as inducers or stimulators of cytodifferentiation and/or antibiotic production.[4,11,12] The structure of pamamycin-607 differs entirely from the butyrolactone-type stimulators; therefore, pamamycin-607 represents the second type of bioregulators chemically identified in the actinomycetes.

Pamamycin-607 showed aerial mycelium-inducing activity at 0.1 μg/disc, but inhibited substrate mycelium at concentrations higher than 10 μg/disc. Preliminary experiments indicate that a pamamycin homologue of MW 621, which comprised three isomers that could not be separated from each other, showed similar aerial mycelium-inducing activity to pamamycin-607. Interestingly, another pamamycin homologue with a MW of 635 also comprised three isomers (but they could be separated from each other) showed only substrate mycelium-inhibitory activity, whereas another of the three showed aerial mycelium-inducing activity. These pamamycin homologues have closely similar structures; therefore, the relation between their structures and their biological activities is of considerable interest. We are now purifying and determining the structures of the other pamamycin homologues.

Pamamycin-607 appeared to be a cation ionophore antibiotic from its macrodiolide structure. However, neither monovalent (K$^+$, Na$^+$) nor divalent (Ca^{2+}) cation was transferred by pamamycin-607 in the *in vitro* two-phase partition system. In contrast, when KMnO$_4$ was partitioned between

benzene and water, only MnO_4^- was transferred from water to the benzene phase by pamamycin-607 under neutral and acidic but not under alkaline conditions.

The anion transfer ability of pamamycin-607 is clearly different from the abilities of cation ionophores such as valinomycin, but it is similar to that of an anion phase transfer catalyst, tetra-*n*-butyl-ammonium chloride. Transfer of Cl^- was also shown by an experiment in which pamamycin-607 dissolved in EtOAc was not extracted into water by shaking with 0.1 N HCl, clearly indicating that a salt form of pamamycin-607 hydrochloride remained in the EtOAc layer. Pamamycin-607 is thus shown to be an unique anion transfer antibiotic not heretofore found in microbial products.

CHOU and POGELL[13] reported that the primary target of pamamycins in growth inhibition of bacteria was the inhibition of phosphate transport. We have now shown that the antibiotic action as well as aerial mycelium-inducing activity of pamamycin-607 should be investigated in relation to its anion transfer activity. Detailed studies on the anion transport abilities of various biologically important anions are now under investigation in hopes of clarifying the interesting biological activity of pamamycin-607.

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