PAMAMYCIN: A NEW ANTIBIOTIC AND STIMULATOR OF AERIAL MYCELIA FORMATION

PAMELA A. MCCANN* and BURTON M. POGELL

Department of Microbiology, St. Louis University School of Medicine,
St. Louis, Missouri 63104, U.S.A.

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Pamamycin is a new antibiotic isolated from *Streptomyces alboniger* ATCC 12461. The antibiotic is active *in vitro* against Gram-positive bacteria, *Neurospora*, and *Mycobacteria*. The compound also acts as a streptomycete differentiation effector. It stimulates aerial mycelia formation in the producing organism. The new antibiotic of elemental composition $C_{36}H_{63}NO_7$ is completely different from puromycin, also produced by this strain. The present communication deals with the isolation, properties, and preliminary characterization of pamamycin.

The regulation of aerial mycelia formation in streptomycetes can be specifically influenced by selective biologically active compounds. Screening of various antibiotics revealed that lincomycin, an inhibitor of protein synthesis produced by *Streptomyces lincolnensis*, exerted differential concentration-dependent effects on *S. alboniger*. When assayed by disc diffusion on agar, very low quantities of the antibiotic ($0.002 \sim 1$ μg per disc) caused a marked enhancement of aerial mycelia formation, visualized as an increase in white powder. In contrast, higher levels ($2 \sim 10$ μg per disc) of the same antibiotic caused aerial mycelia formation to be completely repressed. Although there was no obvious effect on vegetative growth at these levels, white powder never formed. Vegetative growth began to be inhibited when amounts of lincomycin greater than 10 μg were tested. Thus, simply altering the concentration of lincomycin produced a gradient of effects on mycelia development—complete growth inhibition at higher levels, specific inhibition of aerial mycelia formation at intermediate levels and finally, specific stimulation of aerial mycelia development at very low levels. Chloramphenicol, another streptomycete protein synthesis inhibitor, exerted analogous concentration-dependent effects. However, numerous other antibiotics had no obvious specific effects on mycelial development.

These observations led to a search for endogenous differentiation effectors in *S. alboniger*. Several compounds have been isolated which differentially stimulate or inhibit aerial mycelia formation in this organism. The purification, properties, and preliminary characterization of one such compound, pamamycin, are presented here. Pamamycin was isolated from agar-grown cells and specifically stimulates aerial mycelia production. In addition to its role as a streptomycete differentiation effector, pamamycin has high antimicrobial activity. This compound represents a new family-type antibiotic, and is active against Gram-positive bacteria, *Mycobacteria*, and *Neurospora*.

Experimental

The antibiotic activity of pamamycin was assayed by a disc-diffusion assay on tryptic soy agar (TSA) (Difco) against *Sarcina lutea*. Samples were applied to paper discs (6.35 mm diameter) and the dried discs placed on inoculated plates. Plates were kept at 4°C for at least 4 hours prior to incubation. A unit was defined as the amount of pamamycin producing a zone of 17 mm diameter. Activities at

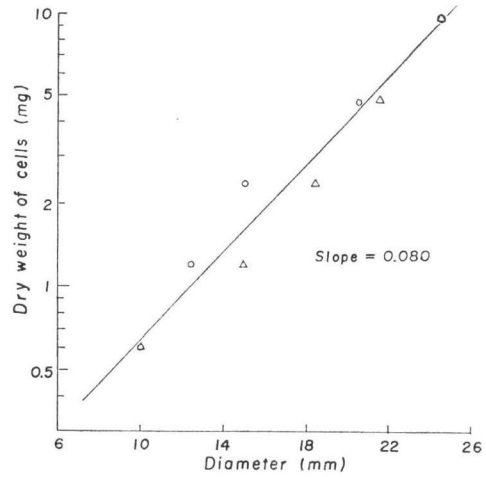
* Present address: Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065, U.S.A.

various stages of purification are expressed as units/mg of dry weight (u/mg).

The aerial mycelia stimulator activity of pamamycin was assayed by a similar disc assay on 2% Bacto-agar (Difco) containing 1% Amidex (Corn Products, Argo, Illinois), 0.2% N-Z-amine Type A (Humpko Sheffield, Norwich, New York), 0.05% yeast extract (Difco), 0.05% beef extract (Difco), and adjusted to pH 7.2. The plates were inoculated by swabbing with an aliquot of a culture of *S. alboniger* grown in HICKEY-TRESNER (HT) medium[1]. After 24~48 hours of incubation at 37°C the zones of aerial mycelia stimulation were visible as circular areas of intense white powder formation. A plot of zone diameter versus log of pamamycin concentration gave a linear response curve (Fig. 1). A unit was defined as the amount of pamamycin producing a zone of 17-mm diameter.

Fig. 1. Quantitation of stimulation of aerial mycelia formation in *S. alboniger* by pamamycin. See Experimental for details.

Each point represents the average of two assays. The concentration has been correlated to the equivalent weight of dried cells represented in the amount of extract assayed at each point.



Aerial mycelia inhibitor activity was also measured by disc assay against *S. alboniger* on agar composed of HT minus $CoCl_2$. This deletion enhanced the contrast between the zones of inhibited aerial mycelia and the white powdery background. The zones were visualized most effectively if the plates were incubated at 37°C for 16~18 hours, and then transferred to 28°C for an additional 12~24 hours. The two inhibitor fractions which were isolated by silicic acid chromatography gave different slopes when plotted as a function of the log of inhibitor concentration versus zone diameter of inhibited aerial mycelia formation. A unit of inhibitor A was arbitrarily defined as a zone of 17-mm diameter and a unit of inhibitor B as a 23-mm zone.

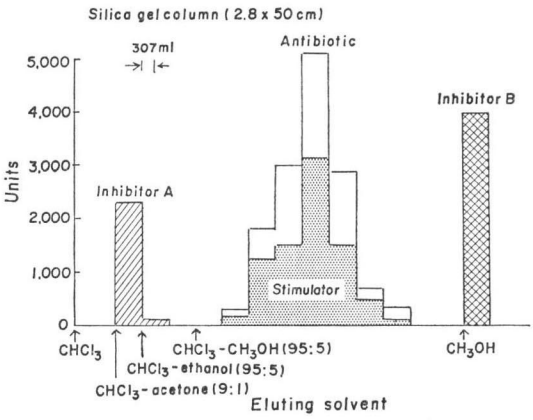
Isolation

Pamamycin was obtained from *S. alboniger* ATCC 12461 mycelia grown on HT agar. Glass Petri dishes (150×15 mm) containing 70 ml agar overlaid with sterile dialysis membrane (A. Thomas, molecular weight cutoff 8,000) were inoculated by swabbing from a HT broth culture (grown 3 days at 28°C, with shaking). After 4~5 days incubation at 28°C, the mycelia were scraped off the membrane, freeze-dried, and stored at −20°C.

Mycelia were extracted with methanol in a Soxhlet apparatus and the extracts concentrated by rotary evaporation. The extracted material from 20 g (dry weight) of mycelia was then triturated into a non-polar solvent, either hexanes or benzene, to obtain 580 mg of a substantially purified extract containing pamamycin. This

Fig. 2. Separation of pamamycin from the inhibitors of aerial mycelia formation.

This elution profile was obtained when 400 mg of hexane-soluble material (extracted from 15 g cells) was chromatographed on a 2.5×50 cm silica gel column. Elution was carried out in 2 V batches as shown.



step was carried out in a 50°C water bath. The pooled extracts were concentrated under N_2 gas and any insoluble particles removed by centrifugation at room temperature in a clinical centrifuge (1.29 units/mg).

Silicic Acid Column Chromatography

This step was used to separate pamamycin from two inhibitors of aerial mycelia formation. Silicic acid (Mallinckrodt CC-4, special for column chromatography) was activated by oven-drying at 105°C for 12 hours, cooled in a desiccator, and used within 24 hours. The column was poured in chloroform, using 150 g silicic acid per g of sample. Chromatography by stepwise elution was carried out as illustrated in Fig. 2, and each eluant fraction concentrated by rotary evaporation. The residues were dissolved in small volumes of toluene and assayed for antibiotic, stimulator, or inhibitor activity as described under Experimental. Active fractions of pamamycin (37 mg) were pooled for further purification (432 units/mg).

Aluminum Oxide Column Chromatography

Aluminum oxide (Woelm Neutral, Brockman Grade I) was deactivated to Grade V by shaking with glass-distilled-deionized water (15 ml per 100 g alumina) and then allowed to stand for 2 hours in a closed container. All solvents used from this point on were freshly redistilled. The pooled silica gel column fractions containing pamamycin activity were concentrated to 150 mg/ml benzene and loaded on the column at a ratio of 1 g of sample per 100 g alumina. Stepwise elution with benzene - chloroform (70: 30, then 60: 40) eluted 17.6 mg of relatively pure pamamycin (852 units/mg).

TLC on Aluminum Oxide

Glass plates (EM, type T, F-254, 250 μ layer) were used for the final step in pamamycin purification. Each plate was prerun in the desired solvent system, air-dried, and then partially deactivated by developing in acetone - water (90: 10). After air-drying, the plate was used within 24 hours. Alumina column-purified samples were pooled and spotted on the TLC plate at a concentration of at least 0.4 mg/area of 1 cm diameter. For final purification, plates were developed in ethyl acetate - methanol (50: 50). Active fractions (Rf$\sim$0.5; 4.9 mg) were eluted with methanol, triturated into toluene, and if necessary rerun in the same solvent system for final purification. The final toluene extract was then washed with water to remove small amounts of impurities. Peak fractions were filtered through a fine sintered glass plate and used for structural analysis (1,100 units/mg). Similar levels of activity (1,000$\sim$1,500 units/mg) were found in several other preparations.

Properties

Pamamycin is insoluble in water but soluble in a wide range of organic solvents including hexanes, ether, benzene, chloroform, methanol, and dimethyl sulfoxide. It behaved as a neutral compound—the activity could not be extracted from chloroform or dichloromethane by 0.1 or 0.5 M acid or base. The mobilities in a variety of TLC systems are given in Table 1. Heating dry pamamycin above 100°C destroyed the activity; after 1 hour at 150°C only 50% of the activity remained. Although purified pamamycin was stable when stored at 4°C as a toluene solution, it slowly lost activity when stored dry. As much as 14% of the activity was lost after 9 days of storage, and 31% after 21 days.

The *in vitro* antimicrobial spectrum of pamamycin is summarized in Table 2. The antibiotic was highly active against Gram-positive bacteria, *Neurospora crassa*, and *Mycobacteria*, but inactive against Gram-negative bacteria.

Table 1. Thin-layer chromatography of pamamycin.

Solvent system	Rf*	
	Alumina	Silica gel
Chloroform	—	0
Benzene - ethyl acetate (70: 30)	0.09	—
Ethyl acetate	0.11	—
Chloroform - methanol (90: 10)	—	0.12
Benzene - methanol (55: 45)	—	0.58
Chloroform - methanol (75: 25)	—	0.56
Butanol - acetic acid - H_2O (3: 1: 1)	—	0.76
Ethyl acetate - methanol (50: 50)	0.5	—

* Rf values obtained on glass plates.

Table 2. Antimicrobial spectrum of pamamycin.

Organism	Diameter of inhibition zone (mm)[a]	
	2 units	4 units
Sarcina lutea	21	25
Bacillus subtilis	10	14
Staphylococcus aureus	10	14
Proteus mirabilis	—	<7
Proteus morganii	—	<7
Escherichia coli	—	<7
Mycobacterium phlei	21[b]	—
Mycobacterium smegmatis	23[b]	—
Neurospora crassa	15[c]	21[c]

[a] Assayed on TSA unless otherwise indicated.
[b] Assayed on DAVIS minimal medium[2].
[c] Assayed on VOGEL's Medium N[3] plus 1% sucrose, 4% sorbose, and 1.5% agar.

Characterization

The molecular weight of purified pamamycin was determined to be 621 by field desorption mass spectroscopy. The mass spectrum (Fig. 3) indicated the presence of possible homologues with added methylene groups at masses of 635, 649, and 663. The elemental composition of the 621 mass ion ($C_{36}H_{63}NO_7$) and other major fragments listed in Table 3 were determined by peak matching and by computer analysis of the high resolution mass numbers.

The IR spectrum (Fig. 4) indicated that pamamycin is highly aliphatic and showed the absence of aromatic, -OH, and -NH groups. There were also no amide I and amide II stretch bands (1600~1700 cm^{-1}). Therefore the nitrogen is probably in a tertiary linkage. A peak at 1725 cm^{-1} indicated the presence of carbonyls, but since this peak was not as intense as the CH stretch band at 2880~2960 cm^{-1}, there are probably only one or two carbonyl groups per molecule.

Fig. 3. Mass spectrum of pamamycin.
Spectrum obtained by direct probe in a LKB-900 instrument (10 μg sample).

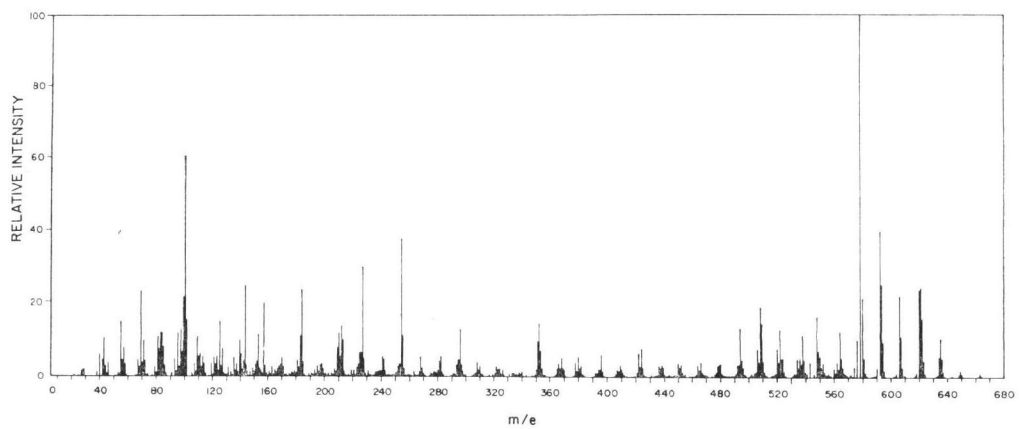
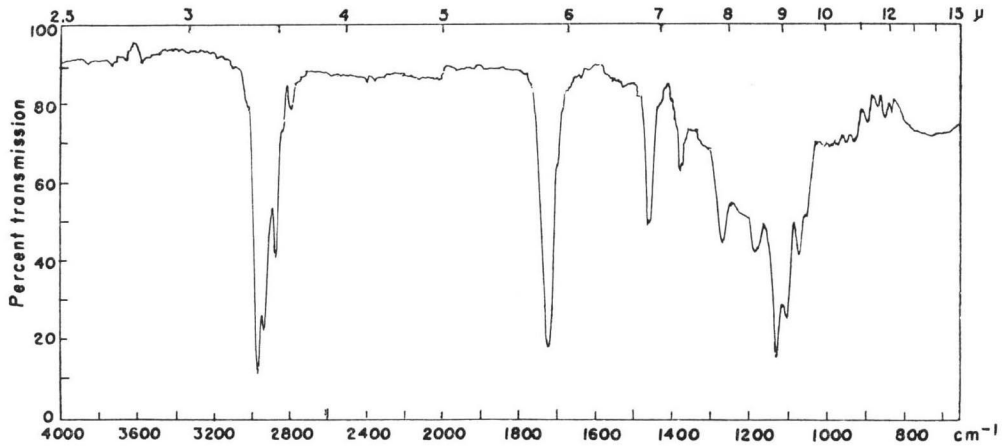


Table 3. Pamamycin fragments obtained by mass spectroscopy

Fragment mass	Molecular formula	Fragment mass	Molecular formula
621	$C_{36}H_{63}NO_7$	254	$C_{16}H_{32}NO$
578	$C_{33}H_{56}NO_7$	227	$C_{13}H_{23}O_3$
508	$C_{30}H_{54}NO_5$	184	$C_{11}H_{22}NO$
352a	$C_{22}H_{42}NO_2$	143	$C_7H_{11}O_3$
352b	$C_{20}H_{34}NO_4$	100	$C_6H_{14}N$

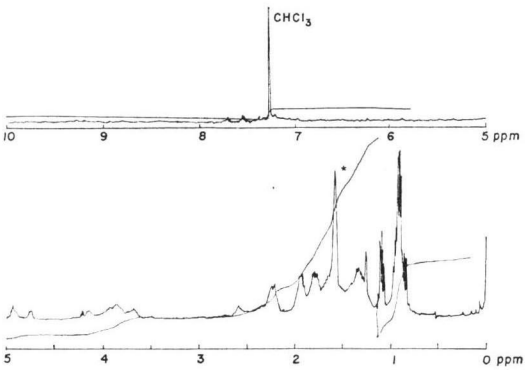
Fig. 4. Infrared spectrum of pamamycin.
Sample was dissolved in chloroform.



NMR (Fig. 5) showed no exchangeable hydrogens, confirming the absence of -OH and -NH groups. The multiplet centered at approximately 0.9 ppm indicates various methyl groups. The multiplets between 1.25~2.0 ppm, and 2.0~2.75 ppm indicate that much of the hydrogen is in methylene and methine groups, respectively. The triplet at 1.1 ppm is consistent with the loss of a propyl group, as seen in the mass spectral fragmentation pattern (fragment 578 results from loss of a propyl group).

Pamamycin showed only end absorption in the UV range, also confirming the absence of conjugated double bonds and aromatic groups.

Fig. 5. NMR spectrum of pamamycin.
Sample was dissolved in $CDCl_3$.



* Due to trace of moisture.

The overall pattern of the IR and NMR suggest that pamamycin is a highly saturated alicyclic compound. A literature search of Chemical Abstracts and other natural product indices and antibiotic screening at Eli Lilly & Co. indicated that there is no previously described compound with this chromatographic behavior, profile of antibiotic activity, and molecular composition.

Discussion

In addition to its antibiotic activity, pamamycin stimulates the formation of aerial mycelia in *S. alboniger*. The wild-type organism will form aerial mycelia in the absence of added pamamycin and quantitative effects of the stimulator were measured as arbitrary zones of increased aerial mycelia formation[4]. Two fractions which inhibited aerial mycelia formation in *S. alboniger* were separated from pamamycin by silicic acid chromatography and could be assayed similarly by measuring zones of decreased aerial mycelia formation[4]. The involvement of the stimulator and inhibitors in the control of aerial mycelia development in *S. alboniger* was supported by analysis of isolates which were permanently unable to form aerial mycelia. These aerial mycelia-negative strains produced no detectable pamamycin but still formed an inhibitor of aerial mycelia formation[4]. The aerial mycelia inhibitors have not yet been further purified or studied.

The separation of two inhibitors of aerial mycelia formation during fractionation of pamamycin on silicic acid probably explains the tremendous increase in yields of antibiotic found after this step, which varied from 150~2000%. Both of these aerial mycelia inhibitor fractions have the unusual property of competitively reversing the inhibition of *Sarcina lutea* growth caused by pamamycin (WEN-GANG CHOU, unpublished observations).

Further chemical and physical studies are necessary in order to determine the structure of pamamycin. It is intriguing that such chemically diverse compounds as lincomycin, A-factor (2-S-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone)[5], and pamamycin all exert similar specific effects on aerial mycelia formation. Other reports have appeared which suggest that specific effectors of both aerial mycelia and spore formation are produced by different streptomycetes[6,7]. The mechanisms of action of these developmental effectors and their diversity of effects on antibiotic and secondary metabolite production will be of extreme interest.

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