Structure-activity Relationship of Pamamycins: Effects of Alkyl Substituents

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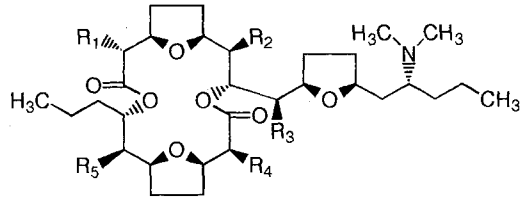
Nine new pamamycin homologues were isolated from the culture broth of *Streptomyces alboniger* IFO 12738 using a combination of ODS and NH_2 HPLCs, and their structures determined by GC-MS. The structural differences in these homologues are in the numbers and positions of methyl and ethyl groups. The aerial mycelium-inducing and growth-inhibitory activities in *S. alboniger* of these homologues and their antibiotic activity against *Bacillus subtilis* were examined. The effects of the alkyl substituents on these activities are discussed.

Pamamycins, a novel family of macrodiolides with MWs ranging from 593 to 663, are produced by *Streptomyces alboniger* IFO 12738[1]. Pamamycin-607 (MW 607) (**1**), a major component of the family, has aerial mycelium-inducing activity in an aerial mycelium-less mutant of *S. alboniger*[1,2]. We determined the structures of four other homologues with MWs of 635 and 649; pamamycin-635A (**2**), -635B (**3**), -649A (**4**) and -649B (**5**). Comparison of these five structures with their aerial mycelium-inducing activities showed that the active homologues such as pamamycin-607 (**1**) and -635B (**3**) have a CH_3-group at R_3, whereas the inactive other three have C_2H_5[3].

To clarify the structure-activity relationship of pamamycins, the effects of other alkyl substituents, R_1, R_2, R_4 and R_5 on aerial mycelium-inducing activity are interesting problems. We, therefore, isolated nine new pamamycin homologues with MWs of 593, 621 and 635, determined their structures and compared their activities with respect to the type of substituent, methyl or ethyl, and their attached positions.

Since pamamycin-607 at higher concentrations has growth inhibitory activity against *S. alboniger* and antibiotic activity against *Bacillus subtilis*[1], the relationships between those activities and alkyl substituents $R_1 \sim R_5$ are also discussed.

Table 1. Structures of pamamycins.



			R_1	R_2	R_3	R_4	R_5	Diols produced by $LiAlH_4$ reduction[a]
Pamamycin-	607	(1)	CH_3	CH_3	CH_3	CH_3	H	L_1, S_1
	635A	(2)	CH_3	CH_3	C_2H_5	CH_3	CH_3	L_1, S_1
	635B	(3)	CH_3	CH_3	CH_3	C_2H_5	CH_3	L_1, S_3
	649A	(4)	C_2H_5	CH_3	C_2H_5	C_2H_5	H	L_2, S_2
	649B	(5)	C_2H_5	CH_3	C_2H_5	CH_3	CH_3	L_2, S_1
	593	(6)	CH_3	H	CH_3	CH_3	H	L_3, S_1
	621A	(7)	CH_3	CH_3	CH_3	CH_3	CH_3	L_1, S_1
	621B	(8)	C_2H_5	H	CH_3	CH_3	CH_3	L_4, S_1
	621C	(9)	CH_3	CH_3	C_2H_5	CH_3	H	L_1, S_1
	621D	(10)	C_2H_5	CH_3	CH_3	CH_3	H	L_5, S_1
	635C	(11)	C_2H_5	CH_3	CH_3	CH_3	CH_3	L_5, S_1
	635D	(12)	C_2H_5	CH_3	CH_3	C_2H_5	H	L_5, S_2
	635E	(13)	CH_3	CH_3	C_2H_5	C_2H_5	H	L_1, S_2
	635F	(14)	C_2H_5	CH_3	C_2H_5	CH_3	H	L_2, S_1

[a]: Structures of the newly isolated pamamycins 6 ~14 were determined by GC-MS analyses of the diols produced by $LiAlH_4$ reduction. See text and Table 2.

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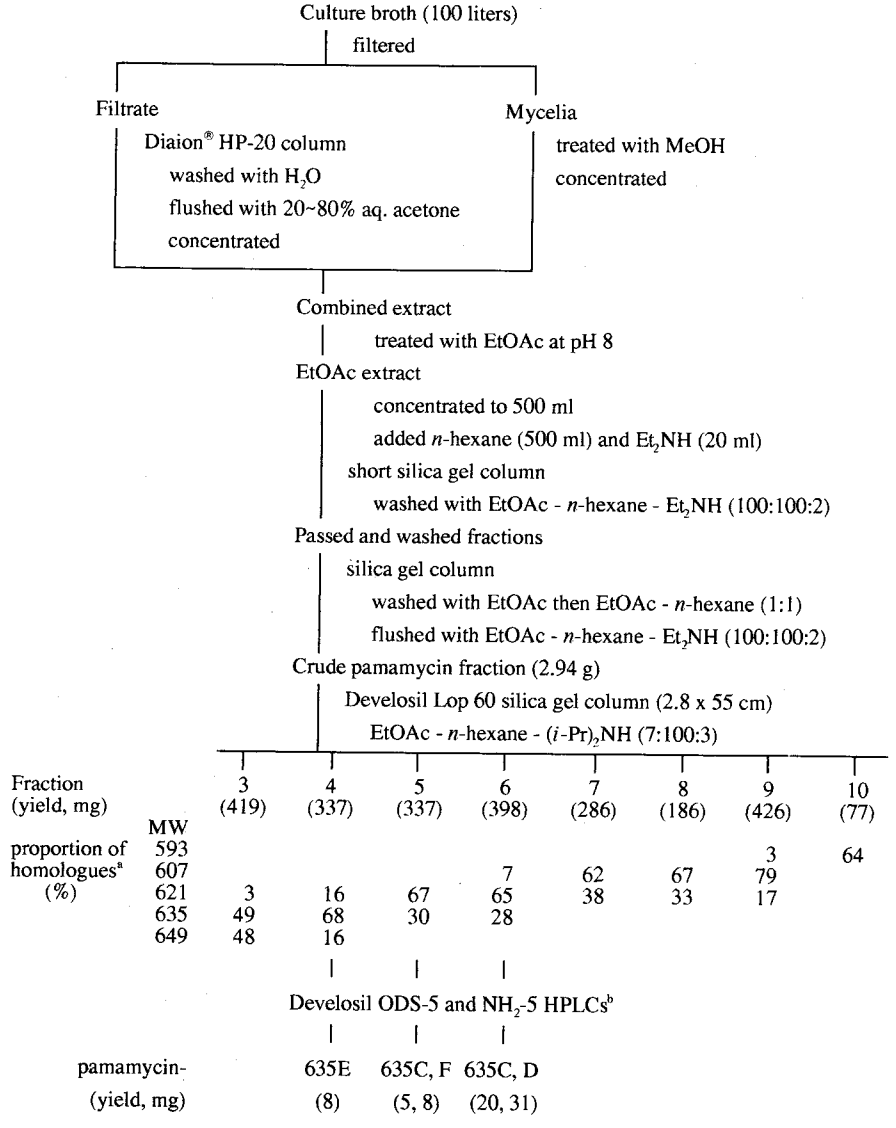
Isolation

The procedure for purifying pamamycin-635 homologues is outlined in Fig. 1. The eluate from a Develosil Lop 60 silica gel column was collected in 11 fractions using HPTLC plate NH_2 and MS analyses[1]. Purification of fraction 6 by Develosil ODS-5 HPLC gave two major peaks (Fig. 2). The earlier eluted peak contained the MW 621 homologue and the later peak the MW 635 homologue. The fraction with the MW 635 homologue was further chromatographed on Develosil NH_2-5 HPLC and two pure pamamycin-635 isomers, pamamycin-635C and -635D, were isolated. Similarly, fraction 5 gave pamamycin-635C and -635F, and fraction 4 pamamycin-635E. Pamamycin-635A and -635B isolated previously[3] were not present in fractions 4~6. Pamamycin-593 and -621A~E were obtained by a similar procedure from another batch of the culture broth.

Structural Elucidation

The structures of the isolated pamamycin homologues were elucidated by GC-MS analysis of the respective diol products obtained by $LiAlH_4$ degradation of each homologue[3]. Pamamycin-621A afforded two diols L_1 and S_1, the former being identical to the larger diol obtained from pamamycin-607 and the latter to the smaller one obtained from pamamycin-635A; hence, its structure was determined to be **7** (Table 1). Pamamycin-593 gave the known S diol and a new L_3 diol. The structure of diol

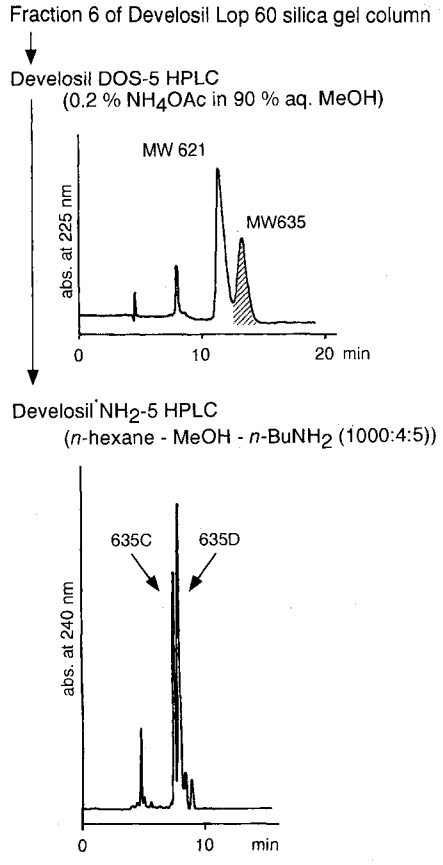
Fig. 1. Purification procedure for pamamycin-635 homologues.



[a] The proportion of the homologues in each fraction was determined from the peak intensity of the M^+ ion in the mass spectrum.

[b] See Fig. 2.

Fig. 2. Purification of pamamycin-635 homologues by HPLC.



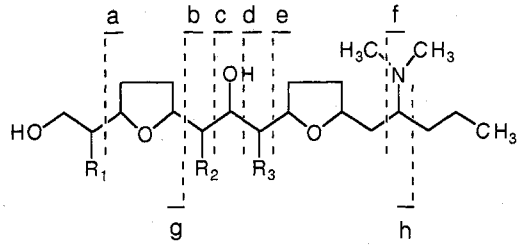
L_3 was determined to be **18** (Table 2) in a comparison of its fragment ions in the MS spectrum with those of diols L, L_1 and L_2, whose structures had been determined previously[3]. The structure of pamamycin-593 therefore was determined to be **6**. The structures of the other homologues isolated were determined by a similar procedure (Tables 1 and 2).

Although the stereochemistries of the newly obtained diols, L_3, L_4 and L_5 were not determined, they should be the same as those of the known diols L, L_1 and L_2, because the coupling patterns of the isolated 8-H signal in the ^1H NMR of all of the newly isolated homologues as well as 2-H of pamamycin-593, -621A and -621C were quite similar to those of respective proton signals of the known pamamycin-607, -635A and -635B (data not shown).

Aerial Mycelium-inducing Activity

Aerial mycelium-inducing activity was assayed by the paper-disc method using an aerial mycelium-less mutant of *S. alboniger* IFO 12738[1]. The activities of the homologues were determined as pamamycin-607 equivalent and are shown as relative values to pamamycin-607 (Fig. 3). Pamamycin-593 had the strongest activity as much as three times of pamamycin-607, and the activity tended to decrease as the MW of the homologue increased. Of the pamamycin-635 isomers, only pamamycin-635C showed activity.

Table 2. Selected mass spectral fragmentation of L series diols.

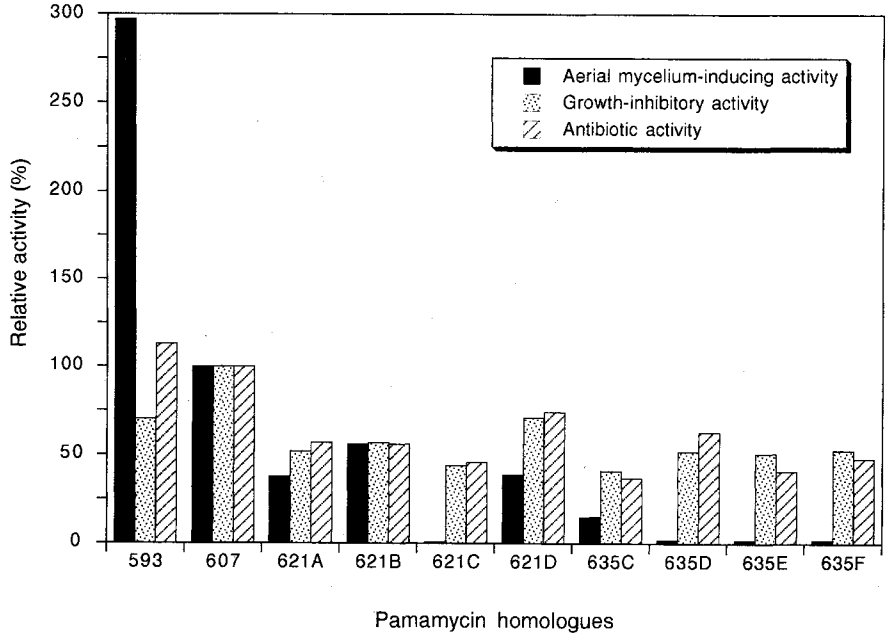


Diol		substituents			fragment ions (*m/z*)								
		R_1	R_2	R_3	M^+	a	b	c	d	e	f	g	h
L	(15)[a]	CH_3	CH_3	CH_3	399[b]	340[b]	270[b]	242[b]	213[b]	184[b]	100[b]	129[b]	356[b]
L_1	(16)[a]	CH_3	CH_3	C_2H_5	413	354	284	256	227	184	100	129	370
L_2	(17)[a]	C_2H_5	CH_3	C_2H_5	427	354	284	256	227	184	100	143	384
L_3	(18)	CH_3	H	CH_3	385	326	256	242	213	184	100	129	342
L_4	(19)	C_2H_5	H	CH_3	399	326	256	242	213	184	100	143	356
L_5	(20)	C_2H_5	CH_3	CH_3	413	340	270	242	213	184	100	143	370

[a] : Structures of diols and their bis-*p*-bromobenzoates were determined from MS and ^1H-NMR spectra[3].

[b] : Cleavage positions were confirmed by HR-MS analyses.

Fig. 3. Relative activities of pamamycin homologues.



Growth-inhibitory Activity against *S. alboniger* and Antibiotic Activity against *B. subtilis*

Growth-inhibitory activity against *S. alboniger* and antibiotic activity against *B. subtilis* also were assayed by the paper disc method. Pamamycin-607 inhibited the growth of *S. alboniger* at more than 10 μg/disc, a 20-fold higher dose than that required for aerial mycelium formation. The MIC of pamamycin-607 against *B. subtilis* is 3.13 μg/ml[1].

The growth inhibitory activities of the newly isolated homologues were 70~40% that of pamamycin-607 (Fig. 3). Antibiotic activity showed a similar tendency, except that pamamycin-593 was slightly stronger than pamamycin-607.

Structure-Activity Relationships

Effects of the alkyl substituents $R_1 \sim R_5$ on the aerial mycelium-inducing activity were examined by comparing the activity of each homologue with that of the homologue in which one substituent R_n differed; *e.g.*, between pamamycin-621D and -607 (R_1 changed), between pamamycin-635C and -621B (R_2 changed) (Fig. 4 left). For R_1, R_2, R_3 and R_5, two pairs were picked up. The mean values of the relative activity of two pairs are defined as the activity coefficient of R_n, which are shown in the graphs in Fig. 4.

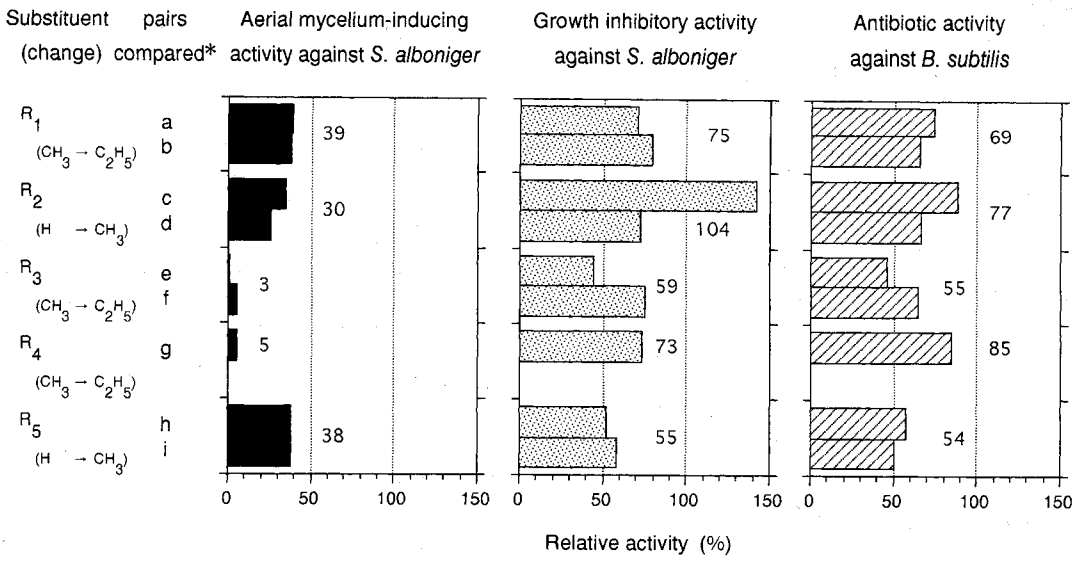
As the results, aerial mycelium-inducing activity mostly was lost when R_3 or R_4 was changed from CH_3 to C_2H_5. Replacement of the other alkyl substituents, R_1, R_2 and R_5, caused a drop to 30~40% of the original activities. We previously showed the importance of R_3 for aerial mycelium-inducing activity by comparing the structures with activities of five pamamycin homologues[3]. We have now confirmed that a change solely of R_3 from CH_3 to C_2H_5 causes a loss of more than 95% of activity and have showed in the present experiment that the same replacement at R_4 resulted in a similar inactivating effect.

When either two of $R_1 \sim R_5$ are changed, the relative activity agrees well with the estimated value calculated by multiplying the activity coefficients of the two Rs changed (Table 3). These results support that the coefficient of each $R_1 \sim R_5$ is reasonable and that the effects of the Rs on aerial mycelium-inducing activity are cumulative.

In consideration with CPK model, pamamycin-607 has the conformation that both R_3 and R_4 extend from one side of the molecule[3]. The importance of this conformation for aerial mycelium-inducing activity has been shown by comparing the activities of pamamycin-607-degraded products (unpublished data). A marked decrease in activity due to change of these two spatially close substituents from methyl to more bulky ethyl group suggests that the expansion of these substituents prevents fitting the molecule into a hole in the active site, so that pamamycin could not exhibit aerial mycelium-inducing activity.

We previously showed that pamamycin-607 needs

Fig. 4. Effects of alkyl substituents on activities of the paramycins.



*a : PM-607 → PM-621D, b : 621A → PM-635C, c : PM-593 → PM-607, d : PM-621B → PM-635C, e : PM-607 → PM-621C, f : PM-621D → PM-635F, g : PM-621D → PM-635D, h : PM-607 → PM-621A, i : 621D → PM-635C.

Numbers in the graphs show mean value of two pairs (except R_4), which are defined as the activity coefficients of Rn.

Table 3. Decrease in aerial mycelium-inducing activity due to two alkyl group changes.

Rs changed	Pairs compared	Relative activity (%)	
		Experimental	Estimated*
1, 2	PM-593 → PM-621D	13	12
1, 3	PM-607 → PM-635G	2	1
1, 4	PM-607 → PM-635D	2	2
1, 5	PM-593 → PM-621B	19	15
	PM-607 → PM-635C	15	
2, 3	PM-593 → PM-621C	1	1
2, 5	PM-593 → PM-621A	13	11
3, 4	PM-635E → PM-607	2	2

* : Estimated activity was calculated by multiplying the two activity coefficients of the Rs changed.

Ca^{2+} for its activity and that calcium signal modulators such as a Ca^{2+} channel blocker verapamil or a calmodulin inhibitor prenylamine inhibit aerial mycelium formation in *S. alboniger*[4]. We recently showed that pamamycin-607 induces a transient increase in the intracellular Ca^{2+} concentration[5]. CHOU and POGELL reported that pamamycin binds tightly to bacterial membranes[6]. These results indicate that pamamycin may act on an ion-translocating mechanism in the cell membrane. We now are investigating the binding site of pamamycin-607, which may regulate the intracellular Ca^{2+} concentration.

Effects of the alkyl substituents $R_1 \sim R_5$ on the growth-inhibitory and antibiotic activities were examined similarly as those on aerial mycelium-inducing activity. The effects of Rs on growth-inhibitory and antibiotic activities obviously differ from the effects on aerial mycelium-inducing activity (Fig. 4 center and right). In every case, expansion of R preserved more than 50% of the original activity and the difference in the R position was small. These results suggest that effect of R on these activities is to change the hydrophobic property of the molecule.

Experimental

Fermentation

A seed culture was grown by transferring a loopful of *S. alboniger* IFO 12738 from an agar slant to five 500-ml shaking flasks, each containing 100 ml of medium composed of 1.5% maltose, 0.3% yeast extract, 1% N-Z Amine A, 0.3% NaCl and 0.2% $CaCO_3$. The inoculated flasks were agitated on a reciprocal shaker for 4 days at 30°C, after which the broth was inoculated to a jar fermentor containing 50 liters of the same medium. Fermentation was carried out for 6 days at 30°C.

Elucidation of the Pamamycin Homologue Structures

Purified pamamycin (50 mg) was allowed to react with $LiAlH_4$ (10 mg) in absolute Et_2O (10 ml) for 3 hours under reflux. Excess $LiAlH_4$ was quenched with a few drops of H_2O, and the reaction mixture passed through a short Na_2SO_4 column then concentrated *in vacuo* to give the diol mixture. This mixture was analyzed with a JEOL JMS-102A GC-MS spectrometer using a DB-1 capillary column (J&W Scientific, 0.25 mm × 15 m, 0.1 mm film thickness).

Bioassay

Aerial mycelium-inducing activity was assayed as reported elsewhere[1]. Pamamycin homologues were assayed at 1, 3 and 30 μg/disc and the diameter of aerial mycelium-inducing zone was converted to pamamycin-607 equivalent by fitting the calibration curve for pamamycin-607. Growth-inhibitory activity against *S. alboniger* was assayed by a procedure similar to that used for aerial mycelium-inducing activity, except that *S. alboniger* IFO 12738 was the test strain used. Antibiotic activity against *B. subtilis* IFO 3134 was examined on nutrient agar medium by the paper-disc method.

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