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 NH₂ 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. 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. 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 CH₃-group at R₃, whereas the inactive other three have C₂H₅ groups. To clarify the structure-activity relationship of pamamycins, the effects of other alkyl substituents, R₁, R₂, R₄ and R₅ 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, the relationships between those activities and alkyl substituents R₁ ~ R₅ are also discussed.
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 NH₂ and MS analyses. 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 NH₂-5 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 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 LiAlH₄ degradation of each homologue. Pamamycin-621A afforded two diols L and S, 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 L₃ diol. The structure of diol

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* The proportion of the homologues in each fraction was determined from the peak intensity of the M⁺ ion in the mass spectrum.

* See Fig. 2.
L₃ was determined to be 18 (Table 2) in a comparison of its fragment ions in the MS spectrum with those of diols L, L₁ and L₂, whose structures had been determined previously. 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, L₃, L₄ and L₅ were not determined, they should be the same as those of the known diols L, L₁ and L₂, 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. 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.

<table>
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<tr>
<th>Diol</th>
<th>substituents</th>
<th>M⁺</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
<th>h</th>
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</thead>
<tbody>
<tr>
<td>L₁</td>
<td>CH₃ CH₃ CH₃</td>
<td>399</td>
<td>340</td>
<td>270</td>
<td>242</td>
<td>213</td>
<td>184</td>
<td>100</td>
<td>129</td>
<td>356</td>
</tr>
<tr>
<td>L₂</td>
<td>CH₃ CH₃ C₂H₅</td>
<td>413</td>
<td>354</td>
<td>284</td>
<td>256</td>
<td>227</td>
<td>184</td>
<td>100</td>
<td>129</td>
<td>370</td>
</tr>
<tr>
<td>L₃</td>
<td>CH₃ H CH₃</td>
<td>399</td>
<td>326</td>
<td>256</td>
<td>242</td>
<td>213</td>
<td>184</td>
<td>100</td>
<td>129</td>
<td>342</td>
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<td>370</td>
</tr>
</tbody>
</table>

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

**: Cleavage positions were confirmed by HR-MS analyses.
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.

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 R₁ ~ R₅ on the aerial mycelium-inducing activity were examined by comparing the activity of each homologue with that of the homologue in which one substituent Rₙ differed; e.g., between pamamycin-621D and -607 (R₁ changed), between pamamycin-635C and -621B (R₂ changed) (Fig. 4 left). For R₁, R₂, R₃ and R₅, two pairs were picked up. The mean values of the relative activity of two pairs are defined as the activity coefficient of Rₙ, which are shown in the graphs in Fig. 4.

As the results, aerial mycelium-inducing activity mostly was lost when R₃ or R₄ was changed from CH₃ to C₂H₅. Replacement of the other alkyl substituents, R₁, R₂ and R₅, caused a drop to 30 ~ 40% of the original activities. We previously showed the importance of R₃ for aerial mycelium-inducing activity by comparing the structures with activities of five pamamycin homologues. We have now confirmed that a change solely of R₃ from CH₃ to C₂H₅ causes a loss of more than 95% of activity and have showed in the present experiment that the same replacement at R₄ resulted in a similar inactivating effect.

When either two of R₁ ~ R₄ 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 R₁ ~ R₄ 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 R₃ and R₄ extend from one side of the molecule. 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
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*. We recently showed that paramycin-607 induces a transient increase in the intracellular Ca\(^{2+}\) concentration. These results indicate that paramycin may act on an ion-translocating mechanism in the cell membrane. We now are investigating the binding site of paramycin-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 \(R_s\) 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$_2$O (10 ml) for 3 hours under reflux. Excess LiAlH$_4$ was quenched with a few drops of H$_2$O, and the reaction mixture passed through a short Na$_2$SO$_4$ 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 x 15 m, 0.1 mm film thickness).

Bioassay

Aerial mycelium-inducing activity was assayed as reported elsewhere. 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.

References


