Inhibition of Microbial Cell Wall Synthesis by Lipopeptin A

Masahiro NISHII, Kiyoshi ISONO* and Kazuo IZAKIF

The Institute of Physical and Chemical Research, Wako-shi,
Saitama 351, Japan

*Department of Agricultural Chemistry, Tohoku University,
Sendai 980, Japan

Received September 3, 1980

Lipopeptin A is a selective inhibitor of in vitro peptidoglycan synthesis of E. coli Y-10. In the study here it inhibited the formation of lipid intermediates from UDP-[U-14C]GlcNAc and UDP-MurNAc-L-Ala-d-Glu-meso-DAP-d-Ala-d-Ala, but did not inhibit the formation of MurNAc-pentapeptide-p-p-lipid from UDP-MurNAc-L-Ala-d-Glu-[3H]meso-DAP-d-Ala-d-Ala. Lipopeptin A also did not have a significant effect on polymerase reaction. Therefore, the inhibition of the formation of GlcNAc-MurNAc-pentapeptide-p-p-lipid from MurNAc-pentapeptide-p-p-lipid and UDP-GlcNAc is concluded to be the site of action.

Lipopeptin A inhibits fungal growth, causing swelling in mycelia. It did not significantly inhibit the incorporations of 14C-labeled glucosamine, thymidine, uridine, phenylalanine, and sodium acetate into TCA insoluble fraction of mycelial suspension of Piricularia oryzae. In in vitro test, however, it inhibited the transfer of mannose from GDP-[U-14C]mannose (ID_{50} = 250 \mu g/ml) and GlcNAc from UDP-[U-14C]GlcNAc (ID_{50} = 100 \mu g/ml) into proteoheteroglycan with a particulate enzyme of Piricularia oryzae. It also slightly inhibited chitin synthesis (ID_{50} = 750 \mu g/ml) in the same enzyme system, but did not inhibit \beta-1,3-glucan synthesis.

In the screening for inhibitors of bacterial peptidoglycan synthesis, lipopeptin A was isolated from the culture filtrates and mycelia of Streptomyces sp. No. AC-69, which resembles Streptomyces violaceochromogenes.1)

Lipopeptin A is a member of peptolide antibiotics having a fatty acid side chain. The structure is shown in Fig. 1. The determination of the structure is reported in a separate paper.2) Amphomycin3) and enduracidin4~6) belonging to this group of antibiotics inhibit peptidoglycan synthesis and the site of action was reported to be the inhibition of the formation of lipid intermediates.7,8)

Lipopeptin A inhibits in vitro peptidoglycan synthesis of Escherichia coli Y-10 but shows only weak inhibitory activity against bacterial cells. On the other hand, it shows inhibitory activity against some species of phytopathogenic fungi inducing characteristic swelling of mycelia.1)

This paper describes the site of action of lipopeptin A in peptidoglycan synthesis of E. coli. In vitro studies on cell wall glycan synthesis of Piricularia oryzae is also described.

\[
\begin{align*}
\text{CH}_3 &\text{-CH}_2 &\text{-CH}_2 &\text{(CH}_2)_10 &\text{-CONH-L-Thr-L-Asp-L-Ser-L-Ser-L-MeAsn-L-MePhe-L-HyGln-L-Glu} \\
&\text{CH}_3
\end{align*}
\]

MeAsn \quad \text{N-methylasparagine}
MePhe \quad \text{N-methylphenylalanine}
HyGln \quad \text{threo-\beta-Hydroxyglutamine}

FIG. 1. Structure of Lipopeptin A.

* Address correspondence to this author.
MATERIALS AND METHODS

Radiochemicals. UDP-[U-14C]GlcNAc, UDP-[U-14C]glucose, GDP-[U-14C]mannose, [U-14C]uridine, [2-14C]thymidine, D-[1-14C]glucosamine, and L-[U-14C]phenylalanine were purchased from Radiochemical Centre, Amersham/Searle. [2-14C]Sodium acetate was purchased from New England Nuclear Corporation. UDP-MurNAc-pentapeptide was prepared as previously described.9)

Organisms and growth conditions. Bacillus cereus T and Escherichia coli Y-10 were grown in bouillon medium (peptone 1%, meat extract 0.3%, NaCl 0.5%, Eiken Chemical Co.) at 37°C on a rotary shaker. Piricularia oryzae was grown in Y.G. liquid medium (yeast extract 1%, peptone 1%, meat extract 0.3%, NaCl 0.5%, Eiken Chemical Co.) at 37°C on a rotary shaker. Piricularia oryzae was grown in bouillon medium (peptone 1%, meat extract 0.3%, NaCl 0.5%, Eiken Chemical Co.) at 37°C on a rotary shaker. Piricularia oryzae was grown in Y.G. liquid medium (yeast extract 1%, peptone 1%, meat extract 0.3%, NaCl 0.5%, Eiken Chemical Co.) at 37°C on a rotary shaker. Piricularia oryzae was grown in bouillon medium (peptone 1%, meat extract 0.3%, NaCl 0.5%, Eiken Chemical Co.) at 37°C on a rotary shaker.

In order to obtain UDP-MurNAc-pentapeptide, B. cereus T was grown in a 30 liter jar fermentor containing 15 liters of bouillon medium.

Preparation of particulate enzyme of E. coli Y-10. Particulate enzyme was prepared by grinding cells of E. coli Y-10 with sea sand (200 ~ 400 mesh) according to the procedure previously reported.9)

Preparation of UDP-MurNAc-L-Ala-D-Glu-meso-DAP-pentapeptide. A reaction mixture containing 3.41 x 10^-3M UDP-[U-14C]GlcNAc and UDP-MurNAc-pentapeptide. A typical incubation mixture contains 5 µmol of Tris-HCl buffer (pH 7.5), 1 m mol of MgCl2, 0.033 µmol of UDP-[U-14C]GlcNAc (Sp.Act. 200 µCi/µmol, 13000 cpm), 10 µmol of UDP-MurNAc-pentapeptide, and 5 µl of the particulate enzyme in a final volume of 50 µl was incubated at 37°C for 10 ~ 120 min. After incubation, the reaction mixture was heated in a steam bath for 1 min and incubated at 37°C for 10 ~ 120 min. After incubation, the reaction mixture was heated in a steam bath for 1 min and then spotted on Whatman 3 MM paper. After descending paper chromatography, radioactivity at the origin was measured.

β-1,3-Glucan synthesis. β-1,3-Glucan synthesis was measured according to the procedure developed recently in our laboratory.12) A reaction mixture containing 3.41 x 10^-3 M UDP-[U-14C]glucose (Sp. Act. 239 µCi/µmol, 15000 cpm), 20 mM sodium cacodylate-HCl buffer pH 6.2, 0.6 mM cellobiose, 2.4 mM MgCl2, and 10 µl of P. oryzae particulate enzyme in a total volume of 50 µl was incubated at 25°C for 1 hr. After incubation, the reaction mixture was boiled for 2 min and then spotted on Whatman 3 MM paper. After descending paper chromatography, radioactivity at the origin was measured.

Formation of MurNAc-pentapeptide-p-p-lipid. The lipid intermediate was prepared by the method described by Tanaka et al.7) A reaction mixture (50 µl) containing 2 x 10^-4 M UDP-MurNAc-L-Ala-d-Glu-[3H]-meso-DAP-d-Ala-d-Ala (Sp. Act. 17.6 µCi/µmol, 12000 cpm), 0.1 M Tris-HCl buffer (pH 7.5), 20 mM MgCl2 and 5 µl of the particulate enzyme was incubated for 10 ~ 30 min. After incubation, the lipid intermediate was extracted with 150 µl of n-butanol-6 µl pyridinium acetate, pH 4.2 (2:1). The extracts were transferred into a scintillation vial and evaporated to dryness. The radioactivity was measured using dioxane scintillation cocktail.

Isolation of GlcNAc-MurNAc-pentapeptide-p-p-lipid and its utilization for polymerase reaction. The lipid intermediate was isolated by the method described by Anderson et al.10)

For polymerase reaction, 0.1 ml of the lipid intermediate was dried under nitrogen stream and other components of the reaction mixture; 0.1 M Tris-HCl buffer (pH 7.5), 200 µM MgCl2, and 10 µl of the particulate enzyme were added to it to give the final volume of 100 µl. After incubation for 2 hr at 37°C, the reaction mixture was heated for 1 min and spotted on Whatman 3 MM paper. After descending paper chromatography with isobutyric acid-1 N NH4OH (5:3) for 16 hr, radioactivity at the origin was measured.
of 50 µl was incubated at 25°C for 30 min. After incubation, reaction was terminated by adding 100 µl of chloroform–methanol (2:1) and spotted on Whatman 3 MM paper. After descending paper chromatography with isobutyric acid–1 N NH₄OH (5:3) overnight, radioactivity at the origin was measured.

RESULTS

Effect of Lipopeptin A on peptidoglycan synthesis

By a particulate enzyme system of E. coli Y-10, peptidoglycan is synthesized from UDP-MurNac-pentapeptide and UDP-GlcNAc via two lipid intermediates, e.g., MurNac-pentapeptide-β-β-lipid and GlcNAc-MurNac-pentapeptide-β-β-lipid. Effect of lipopeptin A on lipid intermediates and peptidoglycan syntheses was examined with this system.

The time courses of syntheses of lipid intermediate and peptidoglycan from UDP-[U-14C]GlcNAc and UDP-MurNac-pentapeptide are shown in Fig. 2. Lipopeptin A inhibited both syntheses completely at the concentration of 500 µg/ml. At 150 µg/ml of lipopeptin A, both syntheses were inhibited by 50%, as calculated from the initial reaction velocity (Fig. 3). This finding suggests that the inhibition of peptidoglycan synthesis is caused by the inhibition of lipid intermediate formation. Two steps are known in the formation of lipid intermediates. The first is the formation of MurNac-pentapeptide-β-β-lipid and the second is the formation of GlcNAc-MurNac-pentapeptide-β-β-lipid from the former and UDP-GlcNAc. If UDP-GlcNAc is omitted from the reaction mixture, only the first step should proceed without the second step. As clearly shown in Fig. 4, lipopeptin A did not inhibit the formation of the first lipid intermediate, MurNac-pentapeptide-β-β-lipid from UDP-MurNac-(³H]DAP)-pentapeptide. We also tested the effect of lipopeptin A on polymerase reaction using crude GlcNAc-MurNac-pentapeptide-β-β-lipid obtained according to the procedure of Anderson et al.¹⁰

Inhibition of peptidoglycan synthesis by lipopeptin A from this lipid intermediate was found to be very weak, if there was any at all. In the same conditions, vancomycin, a selective inhibitor of this polymerase reaction, showed almost complete inhibition at the concentration of 125 µg/ml (Table I).

These results indicate that lipopeptin A inhibits selectively N-acetylglucosamine transferase, which catalyzes the formation of GlcNAc-MurNac-pentapeptide-β-β-lipid from MurNac-pentapeptide-β-β-lipid and UDP-GlcNAc.

Effect on fungal cell wall synthesis

Lipopeptin A inhibits growth of P. oryzae at the concentration of 150 µg/ml¹³ and the
Fig. 3. Inhibition of Lipid Intermediate and Peptidoglycan Syntheses by Lipopeptin A.

The reaction mixture is the same as that shown in Fig. 2. ○, lipid intermediate (37°C, 10 min); ●, peptidoglycan (37°C, 30 min).

Initial reaction velocity was measured from the radioactivities of peptidoglycan or lipid intermediate formed in 30 min and 10 min respectively, in the absence or presence of various concentrations of lipopeptin A. Percent inhibition = (radioactivity incorporated in the absence of lipopeptin A - radioactivity incorporated in the presence of lipopeptin A/radioactivity incorporated in the absence of lipopeptin A) × 100.

Fig. 4. Effect of Lipopeptin A on the Formation of MurNAc-pentapeptide-D-/?-/?-lipid.

A reaction mixture contained 2 × 10^{-4} M UDP-MurNAc-L-Ala-D-Glu[3H]-meso-DAP-D-Ala-D-Ala (Sp.Act. 176 μCi/μmol, 12000 dpm), 0.1 M Tris-HCl buffer pH 7.5, 0.02 M MgCl₂, and 5 μl of E. coli Y-10 particulate enzyme in a total volume of 50 μl. After incubation at 37°C for the time indicated, lipid intermediate was extracted with 150 μl of n-butanol–6 M pyridinium acetate pH 4.2 (2:1) and transferred in a scintillation vial, evaporated and radioactivity was measured.

○, no antibiotic; ●, lipopeptin A 500 μg/ml; ○, lipopeptin A 125 μg/ml.

Table I. The Effect of Lipopeptin A and Vancomycin on Polymerase Reaction

<table>
<thead>
<tr>
<th>Antibiotic added</th>
<th>Radioactivity at the origin (cpm)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Lipopeptin A 500 μg/ml</td>
<td>46</td>
<td>53</td>
</tr>
<tr>
<td>Lipopeptin A 125 μg/ml</td>
<td>116</td>
<td>0</td>
</tr>
<tr>
<td>Vancomycin 125 μg/ml</td>
<td>18</td>
<td>82</td>
</tr>
</tbody>
</table>

swelling of mycelia was observed at little below this concentration.

This indicates that the mechanism of action of this antibiotic is related to the inhibition of cell wall biosynthesis. For example, selective inhibitors of chitin synthetase, polyoxins caused swelling of mycelia and spores of phytopathogenic fungi. Therefore, we tested the effect of lipopeptin A on incorporation of glucosamine, a component of fungal cell wall, into TCA insoluble fraction of mycelial suspension of Piricularia oryzae. As shown in Fig. 5, lipopeptin A inhibited the incorporation of D-[1-{14}C]glucosamine only slightly at the concentration of 1 mg/ml. Incorporation of other radiochemicals, e.g., [U-{14}C]uridine, [2-{14}C]thymidine, L-[U-{14}C]phenylalanine, and [2-{14}C]sodium acetate were also not significantly inhibited (data not shown).

Next, we tested the effect of lipopeptin A on in vitro synthesis of cell wall components of P. oryzae using a particulate enzyme system. Nakajima et al. reported that the cell wall of P. oryzae constitutes of at least three different polysaccharides; proteo-heteroglycan containing D-mannose, D-galactose, β-1,3-glucan, and chitin. Of the cell wall components, β-1,3-glucan occupies more than 60%, while the contents of heteroglycan and chitin are 10% and 13% respectively.
Lipopeptin A Inhibiting Cell Wall Synthesis

Fig. 5. Effect of Lipopeptin A on the Incorporation of Glucosamine into TCA Insoluble Fraction of *Piricularia oryzae*.

At 30 min incubation after the addition of lipopeptin A into the mycelial suspension, D-[l-14C]glucosamine 0.1 μCi (5.88 μCi/μmol) was added and the incubation was started.

O, no antibiotic; ●, lipopeptin A 1 mg/ml; ○, lipopeptin A 100 μg/ml.

Fig. 6. Effect of Lipopeptin A on Chitin Synthesis.

Each reaction mixture contained 0.6 mM UDP-[U-14C]GlcNAc (Sp. Act. 300 μCi/μmol), 16.7 mM Tris-maleate-NaOH buffer pH 7.2, 2.7 mM MgCl₂, 0.6 mM β-mercaptoethanol, 0.06 mM EDTA, 17 mM GlcNAc, antibiotic as indicated, and 10 μl of *P. oryzae* particulate enzyme in a total volume of 60 μl. Incubation was conducted at 25°C for 2 hr. The arrow shows the concentration for 50% inhibition.

Lipopeptin A inhibited β-1,3-glucan synthesis only by 17% at the concentration of 1 mg/ml. This result indicates that the primary site of action of lipopeptin A is not the inhibition of synthesis of β-1,3-glucan, major component of cell wall.

Lipopeptin A inhibited chitin synthesis at high concentration (Fig. 6). Concentration required for 50% inhibition was estimated to be 750 μg/ml. This is a much higher value compared to the minimal inhibitory concentration (150 μg/ml) in *in vivo* testing.

Although details of fungal proteo-heteroglycan synthesis is not known, we studied the incorporation of sugars from GDP-mannose and UDP-GlcNAc into proteo-heteroglycan by a particulate enzyme from *P. oryzae*. The enzyme was found to catalyze the incorporation of mannose from GDP-[U-14C]mannose and GlcNAc from UDP-[U-14C]GlcNAc into proteo-heteroglycan. This reaction is dependent on

Fig. 7. Effect of Lipopeptin A on the Transfer of Mannose from GDP-[U-14C]mannose into Proteo-heteroglycan.

Each reaction mixture contained 1.4 x 10⁻³ mM GDP-[U-14C]mannose (Sp. Act. 146 μCi/μmol), 20 mM sodium cacodylate-HCl buffer pH 6.5, 10 mM MnCl₂, 1 mM β-mercaptoethanol, and 10 μl of *P. oryzae* particulate enzyme in a total volume of 50 μl. Incubation was conducted at 25°C for 60 min.
Fig. 8. Effect of Lipopeptin A on the Transfer of GlcNAc from UDP-[U-14C]GlcNAc into Proteo-heteroglycan.

Each reaction mixture contained the same components as shown in Fig. 7 except 6.7 × 10^{-4} mM UDP-[U-14C]GlcNAc. Incubation was conducted at 25°C for 30 min.

concentration of Mn^{++} (data not shown). As shown in Fig. 7 and Fig. 8, lipopeptin A inhibited the transfer of mannose and GlcNAc and the ID_{50} values were found to be 250 μg/ml and 100 μg/ml, respectively.

From these results, we conclude that lipopeptin A inhibits the synthesis of proteo-heteroglycan and to lesser extent, chitin synthesis. The primary site of action of this antibiotic may be the inhibition of the transfer of GlcNAc into proteo-heteroglycan. Although the total structure of proteo-heteroglycan of P. oryzae is not established yet, it may be that it has diacetylchitobiose structure N-glycosidically linked to an asparagine residue of protein as seen in yeast mannan. If this is the case, the inhibition of transfer of GlcNAc into this moiety by lipopeptin A would also result in the inhibition of transfer of mannose which is linked successively to the diacetylchitobiose.

DISCUSSION

Lipopeptin A has a long chain fatty acid as seen in other antibiotics which inhibit cell wall synthesis, e.g., tunicamycin, enduracidin, amphomycin, and moenomycin, etc. Lipopeptin A resembles enduracidin in view of the lactone structure, in which the hydroxyl group of threonine participate in the lactone formation. In this paper, we showed that lipopeptin A inhibits peptidoglycan synthesis as the results of inhibition of the formation of GlcNAc-MurNAc-pentapeptide-p-p-lipid. Lipopeptin A does not inhibit the formation of MurNAc-pentapeptide-p-p-lipid. Also it does not inhibit polymerase reaction. In view of the structure-activity relationship, it is interesting that both lipopeptin A and enduracidin inhibit the same site. Enduracidin inhibits peptidoglycan synthesis and the site of action is reported to be the inhibition of the formation of GlcNAc-MurNAc-pentapeptide-p-p-lipid.

Lipopeptin A also caused swelling of mycelia of fungi, as seen in polyoxins, selective inhibitors of chitin synthetase. However, lipopeptin A inhibited the incorporation of glucosamine into TCA insoluble fraction of mycelial suspension of P. oryzae only slightly. Incorporations of uridine, thymidine, phenylalanine, or sodium acetate in vivo were also not inhibited.

In in vitro experiment, we attempted to see the effect of lipopeptin A on chitin, β-1,3-glucan, and proteo-heteroglycan syntheses with a particulate enzyme from P. oryzae. β-1,3-Glucan synthesis was inhibited only by 17% at the concentration of 1 mg/ml of lipopeptin A but chitin synthesis was inhibited at the high concentration of the antibiotic (ID_{50}; 750 μg/ml). Recently, Nakajima et al. in the study of cell wall components of P. oryzae, clarified the partial structure of proteo-heteroglycan, which has a carbohydrate moiety composed of D-mannose, D-glucose, and D-galactose in a ratio of 6:2:1. They suggested that proteo-heteroglycan might have two kinds of carbohydrate-protein linkage, namely N-glycosidic and O-glycosidic. In yeast mannan, a heteropolysaccharide moiety is linked via a diacetylchitobiose bridge by an N-glycosidic bond to an asparaginyl residue in the protein part and a short manno oligosaccharide is O-glycosidically linked to serine.
and/or threonine residues in the protein. Most of mannosyl units in the mannan molecule are transferred from GDP-mannose. However, in some cases, the transfer of mannoses to mannan does not occur directly but through lipid intermediate, dolichol phosphate mannan. In the biosynthesis of N-glycosidically linked mannan, N-acetylglucosamine is transferred from UDP-GlcNAc to dolichol phosphate to give dolichol-p-p-GlcNAc and dolichol-p-p-(GlcNAc)₂.

Biosynthesis of carbohydrate moiety of proteo-heteroglycan in P. oryzae is not known and existence of N-glycosidic and O-glycosidic linkages in proteo-heteroglycan is only presumptive. In the present study, we showed that lipopeptin A inhibited the transfer of mannose from GDP-mannose into proteo-heteroglycan. Lipopeptin A was also found to inhibit transfer of GlcNAc from UDP-GlcNAc. In our in vitro system, GlcNAc, the primer of chitin synthesis and Mg²⁺ ion are absent and the concentration of UDP-GlcNAc is very low compared to the Km (1.43 × 10⁻³ M) of chitin synthetase.²⁰ Because chitin synthesis does not occur in this conditions, the transfer of GlcNAc from UDP-GlcNAc may reflect only proteo-heteroglycan synthesis but not chitin. This assumption was supported by the fact that ID₅₀ value for proteo-heteroglycan (100 μg/ml) of lipopeptin A was lower than that for chitin synthesis (750 μg/ml).

In conclusion, the swelling of mycelia of fungi caused by lipopeptin A may be due to the inhibition of cell wall synthesis, especially proteo-heteroglycan synthesis and the site of action is suggested to be the transfer of GlcNAc into proteo-heteroglycan.

This may explain failure of significant inhibition of glucosamine incorporation in vivo by lipopeptin A. If the structure of proteo-heteroglycan of P. oryzae has indeed diacetylchitobiose linked to an asparagine residue of protein as seen in yeast mannan, the chemical content of this N-acetylgalactosamine should be considerably low, thus the inhibition of incorporation in vivo can hardly be observed.

Recently, Kang et al.²¹,²² reported that amphotomycin inhibits the transfer of mannose from GDP-mannose and GlcNAc from UDP-GlcNAc to lipid linked saccharide with enzyme preparations of pig arota. Inhibition of the transfer of GlcNAc is known in tunicamycin, which inhibits the formation of dolichol-p-p-GlcNAc.²³ Lipopeptin A has a long chain fatty acid as seen in these two antibiotics; thus, the inhibition of proteo-heteroglycan synthesis by this antibiotic may suggest the presence of lipid intermediate in cell wall glycan synthesis in P. oryzae.

Acknowledgment. We are grateful to Dr. T. Nakajima of Tohoku University for his valuable discussion and helpful advice. We also thank Mrs. R. Nishii for her technical assistance.

REFERENCES
12) T. Kusano and K. Isono, manuscript in preparation.