Rapid Communication J. Biochem. 119, 226-230 (1996)

Structure Determination of an Immunopotentiator Peptide, Cinnamycin, Complexed with Lysophosphatidylethanolamine by ^{1}H-NMR[1]

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Received for publication, October 30, 1995

The three-dimensional structure of a complex of cinnamycin, a 19-amino acid residue immunopotentiator peptide, and lysophosphatidylethanolamine was determined by ^{1}H-NMR. The complex was cylindrical in shape, 11 Å in diameter and 26 Å in length, excluding the acyl chain of the phospholipid. The peptide had a hydrophobic pocket surrounded by residues Phe-7 through Ala(S)-14 to bind to the head group of the ligand. Fitting of the head group to the hydrophobic pocket was so good that other than a glycerophosphoethanolamine head group would be unable to fit the pocket. The goodness of the fitting is compatible with the strict specificity of ligand binding of the peptide.

Key words: cinnamycin, lysophosphatidylethanolamine, nuclear magnetic resonance, peptide-lipid interaction, three-dimensional structure.

Cinnamycin (also known as Ro09-0198) is an immunopotentiator secreted by *Streptoverticillium griseoverticillatum*, possessing anti-tumor and anti-microbial activities (*1*). It is a nonadecapeptide containing various uncommon amino acids, *e.g.*, lanthionine, β-methyllanthionine, lysinoalanine, and β-hydroxyaspartic acid, the first three of which constitute intramolecular bridges (Fig. 1) (*2-4*). Though cinnamycin is a rather short peptide, comprising only 19 amino residues, it strictly recognizes and binds to the phosphatidylethanolamine (PE) or lysophosphatidylethanolamine (lysoPE) structure. Cinnamycin itself makes PE- or lysoPE-containing vesicles leaky (*5*), and its biotinylated derivative, retaining high specificity to ligand binding, has been used as a probe for the transbilayer movement of PE (*8*).

It would be of importance to elucidate the ligand recognition mechanism of cinnamycin. A general requirement for a lipid to be a ligand of cinnamycin is the presence of a lysoPE moiety, *i.e.*, (i) a free primary amino group, (ii) three carbon atoms at a glycerol moiety, and (iii) a hydrophobic chain. Also needed for increasing lipid bilayer permeability, in particular, is (iv) the presence of an oxygen atom at C^2 of the glycerol moiety (*5, 6*). We have already carried out a ^{1}H-NMR study on cinnamycin, finding that (i) β-hydroxyaspartic acid-15 is involved in the binding to the free amino group of lysoPE, (ii) the hydrophobic residues, Gly-8, Pro-9, and Val-13, are involved in the binding to the glycerol moiety, and (iii) the fatty acyl chain is not in contact with the cinnamycin molecule (*7*).

The conformations of cinnamycin molecules in an aqueous solution and SDS-micelles have been determined by ^{1}H-NMR (*4, 8*), but those of the peptide-ligand complex have not been reported so far. To deepen our understanding of the mechanism of ligand recognition of cinnamycin, we report for the first time in this paper the three-dimensional structure of the cinnamycin-lysoPE complex elucidated by means of a ^{1}H-NMR study and distance-geometry calculations followed by simulated annealing.

Cinnamycin was a generous gift from Dr. Kenji Takemoto, Nippon Roche, and Professor Keizo Inoue, the University of Tokyo. 1-Dodecanoyl-*sn*-glycerophosphoethanolamine (C12-lysoPE) was purchased from Avanti Polar-Lipids (Alabaster, AL). Proton NMR spectra (at 500 MHz) were recorded with a Bruker AMX-500 spectrometer at 45°C. The cinnamycin-C12-lysoPE complex was dissolved in $(CH_3)_2SO$-d_6 at 10 mM. Two-dimensional DQF-COSY (*9*), NOESY (*10*), P.E.COSY (*11*), and ROESY (*12*) experiments were performed in the phase-sensitive mode using a time proportional phase increment (*13*). The mixing time was set at 100, 200, and 300 ms for NOESY experiments, and 100 and 200 ms for ROESY experiments. The time domain data size was 1K points in t_1 and 8K points

[1] This research was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, and by a grant from the Japan Securities Scholarship Foundation.

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Abbreviations: Ala(S)Ala, lanthionine [NH-CH(CO)-CH_2-S-CH_2-CH(CO)-NH]; Abu(S)Ala, β-methyllanthionine [NH-CH(CO)-CH(CH_3)-S-CH_2-CH(CO)-NH]; C12-lysoPE, 1-dodecanoyl-*sn*-glycerophosphoethanolamine; C2-lysoPE, 1-acetyl-*sn*-glycerophosphoethanolamine; DQF-COSY, double-quantum-filtered correlation spectroscopy; HO-Asp, β-hydroxyaspartic acid [NH-CH(CO)-CH(OH)-COOH]; HOHAHA, homonuclear Hartmann-Hahn correlation spectroscopy; Lys(N)Ala, lysinoalanine [NH-CH(CO)-$(CH_2)_4$-NH-CH_2-CH(CO)-NH]; lysoPE, lysophosphatidylethanolamine; NOESY, nuclear Overhauser enhancement spectroscopy; PE, phosphatidylethanolamine; P.E.COSY, primitive exclusive correlation spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy.

in t_2 for P.E.COSY, and 512 points in t_1 and 2K points in t_2 otherwise. For each t_1 value, 64 transients were accumulated. Before Fourier transformation, time domain data were multiplied by a shifted squared sine bell for both the t_1 and t_2 dimensions. Zero-filling was employed to give the frequency domain data size 1K × 4K for P.E.COSY, and 512 × 2K otherwise.

Proton resonance assignments of the cinnamycin-C12-lysoPE complex have been previously reported (*7*). Stereospecific assignments of non-equivalent C^{β}-protons were carried out by referring to the intraresidue NOEs of $C^{\alpha}H$-$C^{\beta}H$ and NH-$C^{\beta}H$, and to the $^3J_{\alpha-\beta}$ coupling constants determined in a P.E.COSY experiment, as reported (*14*). NOE restraints were extracted from the NOESY spectrum with the mixing time of 200 ms, which were subsequently classified into four distance restraints: ≤3, ≤4, ≤5, and ≤6 Å, corresponding to strong, medium, weak, and very weak intensity of NOESY cross peaks, respectively. For the NOEs involving methyl groups, pseudoatoms were introduced at the center of mass of the three methyl protons, and distance correction was performed according to Wüthrich *et al.* (*15*). The ROESY spectra with mixing times of 100 and 200 ms showed cross-peaks due to chemical exchange between Gly-8 NH and HO-Asp-15 NH, and to the indirect transfer (spin diffusion) effect between Ala(N)-6 $C^{\alpha}H$ and Phe-7 $C^{\alpha}H$, Ala(S)-5 $C^{\alpha}H$ and Ala(S)-5 $H^{\beta 3}$, Ala(S)-5 $H^{\beta 2}$ and Abu(S)-11 H^{β}, Gly-8 H^{α} and Pro-9 $H^{\delta'}$, Gly-8 $H^{\alpha'}$ and ethanolamine POCH, and Gly-8 H^{α} and ethanolamine POCH′ (a prime sign denotes that, of the two methylene protons, the primed one resonates in the upper field); these cross-peaks were disregarded in subsequent calculations. Thus, 139 intramolecular distance restraints (71 intraresidue, 43 sequential, and 25 medium- and long-range ones) and 33 intermolecular NOE restraints were obtained. Seven restraints on angle ϕ [for Gln-3, Ala(S)-4, Ala(N)-6, Phe-7, Phe-10, Phe-12, and Asn-17] and five on angle $\chi 1$ (for Phe-7, Phe-10, Phe-12, Val-13, and Lys-19) were also introduced, according to Wagner *et al.* (*14*). The bond geometries of uncommon amino acid residues were constructed using a MOLEDIT program (JEOL, Tokyo).

Structure calculations were carried out in two stages. In the first stage, distance geometry calculations in the dihedral angle space (*16*) were performed using a DADAS90 program ver. 4.3 (*17*) (JEOL). Fifty structures

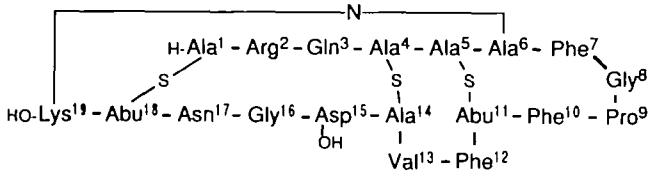

Fig. 1. **Primary structure of cinnamycin.**

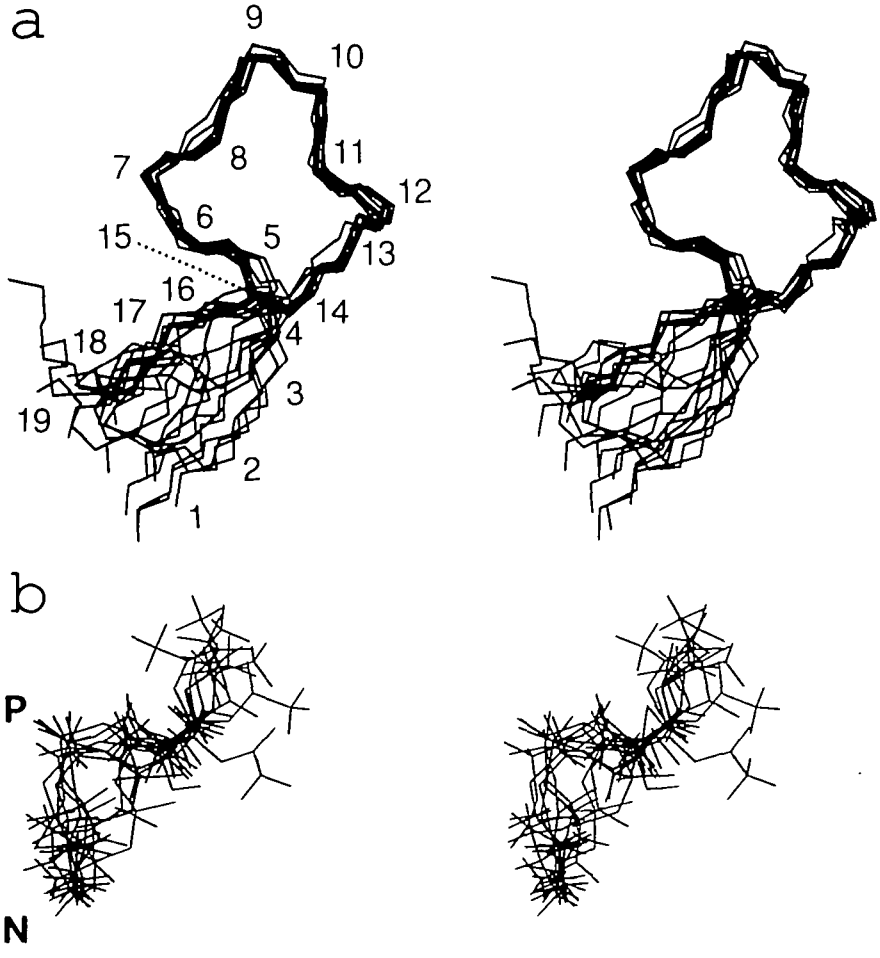

Fig. 2. **Stereopair of the best-fit overlay of 10 refined structures of the cinnamycin-C2-lysoPE complex; main chain of cinnamycin (a) and the glycerophosphoethanolamine moiety (b).**

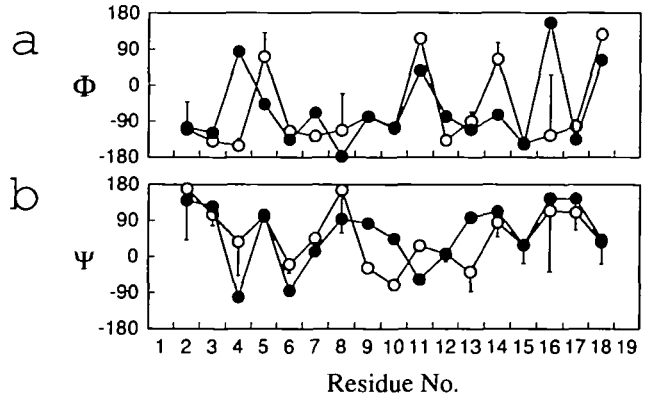

Fig. 3. **Distributions of the ϕ (a) and ψ (b) angles in the amino acid sequence of cinnamycin.** Closed and open circles indicate angles in an aqueous solution (*4*) and a DMSO solution (this study), respectively. Bars represent the standard deviations in a DMSO solution.

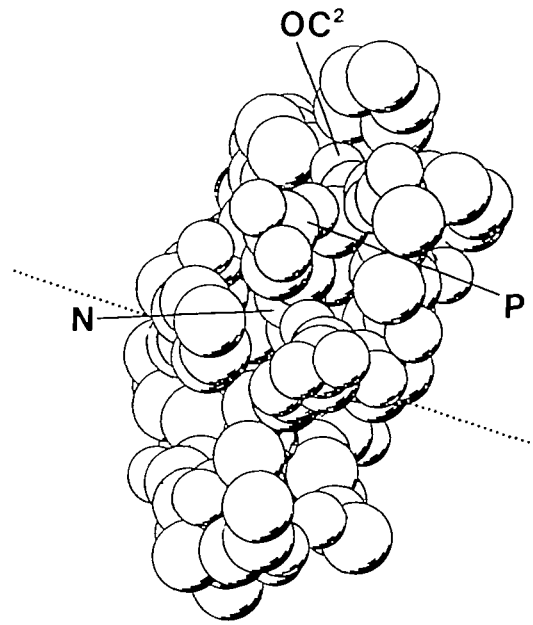

Fig. 4. **Space-filling representation of the mean structure of the cinnamycin-C2-lysoPE complex.** Only heavy atoms are shown. Shaded spheres indicate C12-lysoPE, and OC^2, P, and N annotate the oxygen atom on glycerol C^2, the phosphorus atom, and the nitrogen atom of the ammonium group, respectively.

of the cinnamycin-lysoPE complex were calculated using a simulated annealing protocol and second derivative minimization. The 5 structures having the lowest violation values were selected. In the second stage, 10 structures were calculated, starting from each of the five DADAS90 structures, by means of the simulated annealing (sa.inp) protocol of X-PLOR ver. 3.1 (*18*) using default parameter values. The resultant 50 structures were further refined by the refine.inp protocol and the 10 structures exhibiting the lowest energy were finally selected. For methylene protons that could not be stereospecifically assigned by the above procedure (including those of glycines), we used pseudo-atoms located at the center of mass of the methylene protons, with appropriate distance correction (*15*). Since the acyl chain of C12-lysoPE is known not to interact with a cinnamycin molecule (*7*), C2-lysoPE having an acetyl chain at OC^1 of the glycerol moiety was used instead of C12-lysoPE throughout the calculations. The mean structure was calculated using an XPLOR-UP utility (Kohda *et al.*, unpublished results) according to Lancelin *et al.* (*19*). The final coordinates of each annealed structure were obtained by fitting to the mean structure for the backbone atoms of residues 4-14. RMSDs per residue between the individual final structures and the mean structure were calculated. The molecular structures were analyzed using Insight II (Biosym Technologies, San Diego, CA) and X-PLOR programs. Space-filling representation of the complex was drawn using MOLSCRIPT (*20*).

Figure 2a shows the best-fit overlay of the 10 refined cinnamycin structures in the complex. The structures converged well with minimum atomic RMSDs for backbone atoms (N, C^α, and C′) being 1.05 ± 0.22 and 0.54 ± 0.17 Å for residues 1-19 and residues 3-15, respectively, and with minimum atomic RMSDs for all non-hydrogen atoms being 1.47 ± 0.42 and 1.33 ± 0.39 Å for residues 1-19 and residues 3-15, respectively. The structures of C2-lysoPE in the complex (Fig. 2b) are less defined than those of cinnamycin primarily due to the lack of restraints on the phosphate group and glycerol C^1H_2 protons. The complex structures are considered accurate for the following reasons: (i) None of the NOE restraints were violated by more than 0.25 Å and none of the dihedral restraints were violated, (ii) a Ramachandran plot indicated that each (ϕ, ψ) pair is in an allowed region (not shown; refer to Fig. 3), and (iii) the Lennard-Jones energy of the system is negative (-45 kcal·mol^{-1}), indicating attractive contact between atoms.

The backbone conformation converged well for residues Gln-3 through HO-Asp-15 with backbone RMSD values smaller than 1.0 Å. The lysine-19 side chain, for which two orientations are possible relative to the plane formed by the main chain and (methyl)lanthionine bridges, was found to be exclusively above the plane (Fig. 1); this orientation coincides with that determined previously for a cinnamycin molecule in an aqueous solution (*4*).

The complex is cylindrical in shape, 11 Å in diameter and 26 Å in length, excluding the acyl chain of the phospholipid (Fig. 4). As has been pointed out for a free cinnamycin molecule in an aqueous solution (*8*), hydrophobic residues are clustered in one half of the cylinder (above the dotted line in Fig. 4) and hydrophilic ones in the other half. The cinnamycin molecule in the complex has a hydrophobic pocket formed by residues Phe-7 through Ala(S)-14 which accommodates the glycerophosphoethanolamine head group of C2-lysoPE. This pocket is supported by underlying intramolecular bridges of one lanthionine and two β-methyllanthionine residues. Although the structures of C2-lysoPE are not well defined (Fig. 2b), intermolecular contacts are consistently observed in all the complex structures between ethanolamine CH_2N methylene and Gly-8 methylene, glycerol C^3 methylene and Val-13 $C^{\gamma 2}$ methyl, glycerol C^3 methylene and Pro-9 C^δ methylene, and glycerol C^2 methine and Val-13 $C^{\gamma 2}$ methyl. Besides these hydrophobic interactions, the complex is also stabilized by an ionic interaction between the carboxylate group of HO-Asp-15 and the ammonium group of lysoPE. Intermolecular (as well as intramolecular) hydrogen bonding (*4*,

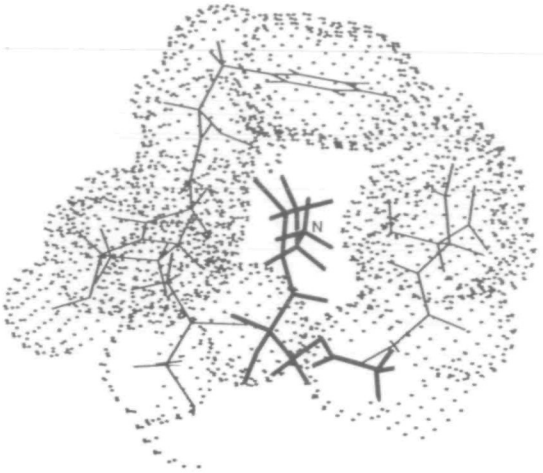

Fig 5. **Expansion of a cinnamycin molecule around the NH_3 group of lysoPE.** The lysoPE molecule is drawn as thick bars, and the residues of cinnamycin around the NH_3 group are shown as clouds and thin bars.

21) and a type-II turn structure (*21*) previously predicted were not found in the present structure. In our earlier study, an ionic interaction was suspected between the secondary ammonium group of lysinoalanine and the phosphate group of lysoPE based on the fact that the ^{1}H-NMR signals of two ammonium protons of lysinoalanine are observable only in the presence of lysoPE (*7*). In the refined complex structure, however, the two groups are 11.7 Å apart and the suspected ionic interaction does not seem to take place. Since the ammonium group of a free cinnamycin molecule in a DMSO solution was found to be close to the carboxylate group of HO-Asp-15 (*8*), the appearance of the two ammonium protons upon the complexation with lysoPE may well be due to the dissociation of these two charged groups.

In agreement with the high specificity of cinnamycin for the glycerophosphoethanolamine moiety, there is no space that can accommodate even a single additional methyl (or methylene) group around the NH_3 group (Fig. 5) or the glycerol moiety (not shown). On the other hand, the hydroxyl group at C^2 of glycerol is exposed to the solvent, hence the cinnamycin molecule can also bind to a PE molecule which has an acyl chain at OC^2 as well as OC^1, in the same conformation, as shown in Fig. 2a.

The main chain dihedral angles (ϕ and ψ) of a free cinnamycin molecule in an aqueous solution (*4*) are compared with those of the cinnamycin complexed with lysoPE in Fig. 3. Distinct differences ($\geq 90°$) can be seen for residues Ala(S)-4, Ala(S)-5, Pro-9, Phe-10, Val-13, and Ala(S)-14, in agreement with the binding of the phosphoethanolamine moiety to many of these residues. For a free cinnamycin molecule in an aqueous solution, an extremely high ($\geq 80°$) RMS value was observed of Phe-7 ψ (*8*), denoting the high flexibility at this residue. The absence of such a high RMS value in the complex indicates that the peptide takes a rigid conformation upon complexation with lysoPE. In conclusion, we determined the three-dimensional structure of the cinnamycin-lysoPE complex; the resultant structure explains the strict binding specificity of the peptide to (lyso)-PE.

We are grateful to Dr Kenji Takemoto, Nippon Roche, and Professor Keizo Inoue, the University of Tokyo, for the generous gift of cinnamycin, and to Dr. Daisuke Kohda of the Tokyo Metropolitan Institute of Medical Science for calculation of the mean structure with the XPLOR-UP program.

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