Torchia, D. A., Sparks, S. W., & Bax, A. (1989) *Biochemistry 28*, 5509–5524.
Tucker, D. W., Hazen, E. E., Jr., & Cotten, F. A. (1978) *Mol. Cell. Biochem. 22*, 67–77.
Tucker, D. W., Hazen, E. E., Jr., & Cotten, F. A. (1979) *Mol. Cell. Biochem. 23*, 3–16, 67–86, 131–141.
Wagner, G., Neuhaus, D., Wörgötter, E., Vasak, M., Kagi, J. H. R., & Wüthrich, K. (1986) *J. Mol. Biol. 187*, 131–135.
Wang, J., LeMaster, D. M., & Markley, J. L. (1990) *Biochemistry* (preceding paper in this issue).
Wüthrich, K. (1984) *J. Mol. Biol. 180*, 715–740.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.

Complex Formation of Peptide Antibiotic Ro09-0198 with Lysophosphatidylethanolamine: ^{1}H NMR Analyses in Dimethyl Sulfoxide Solution†

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ABSTRACT: Ro09-0198 is a peptide antibiotic and immunopotentiator produced by *Streptoverticillium griseoverticillatum* which exhibits antitumor and antimicrobial activities. The chemical structure has been determined [Kessler et al. (1988) *Helv. Chim. Acta 71*, 1924–1929; Wakamiya et al. (1988) *Tetrahedron Lett. 37*, 4771–4772]. This peptide specifically interacts with (lyso)phosphatidylethanolamine, causing hemolysis and enhancing permeability in phosphatidylethanolamine-containing vesicles [Choung et al. (1988) *Biochim. Biophys. Acta 940*, 171–179, 180–187]. The highly specific nature of the interaction was studied by two dimensional proton NMR analyses. Proton resonances of the peptide were observed in dimethyl sulfoxide solution in the presence of 1-dodecanoyl-*sn*-glycerophosphoethanolamine. By comparison to the chemical shifts in the absence of lysophosphatidylethanolamine and by analysis of intermolecular cross-peaks in NOESY spectra, amino acid residues involved in the binding with the phospholipid were identified. The ammonium group of the phospholipid interacts with the carboxylate group of β-hydroxyaspartic acid-15 but not with that of the carboxylate terminus. The secondary ammonium group of lysinoalanine-19/6 is probably bound to the phosphate group of the lipid. The peptide does not interact strongly with the fatty acid chain of the lipid. A folded structure of the central part [from Phe^7 to Ala(S)^{14}] of the peptide opens on binding with the phospholipid and accommodates the glycerophosphoethanolamine head group.

Ro09-0198 is a peptide antibiotic and immunopotentiator found in culture fluid of *Streptoverticillium griseoverticillatum* which exhibits antitumor and antimicrovial activity (Takemoto, 1981). Kessler et al. (1987, 1988) determined the chemical structure of the peptide by a combination of chemical methods and a variety of NMR methods. Independently, Wakamiya et al. (1988) determined the peptide structure (including the chirality of amino acid residues) by chemical methods. It is a nonadecapeptide containing various uncommon amino acids, lanthionine, β-methyllanthionine, lysinoalanine, and β-hydroxyaspartic acid, the former three forming intramolecular bridges (Figure 1a).

The amino acid composition of this peptide is quite similar to that of duramycin (Arg^2 is replaced by lysine in duramycin; Shotwell et al., 1958), which suggests similar action mechanisms of these antibiotics. In fact, these peptides have been found to interact specifically with certain lipid components of the cell membrane. Duramycin induces aggregation of lipid vesicles containing phosphatidylethanolamine or monogalactosyldiglyceride (Navaro et al., 1985), while Ro09-0198 induces hemolysis which is blocked by preincubation of the peptide with phosphatidylethanolamine-containing vesicles (Choung et al., 1988a,b). Ro09-0198 is also found to cause leakage of small molecules from vesicles containing phosphatidylethanolamine or lysophosphatidylethanolamine. The interaction of Ro09-0198 with lipids is highly specific, and the structural requirement for the lipid is the presence of the phosphoethanolamine moiety with a free primary amino group and a hydrophobic chain. In addition, the glycerol moiety is required for the permeability increase through the lipid bilayer (Choung et al., 1988a,b).

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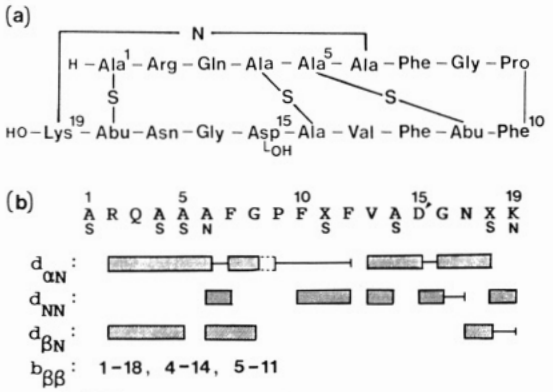

FIGURE 1: (a) Primary structure of Ro09-0198 (Kessler et al., 1988; Wakamiya et al., 1988). (b) Assignments of Ro09-0198 in the presence of C12-LPE. $d_{\alpha N}$, d_{NN}, and $d_{\beta N}$ are for sequential NOEs for proton pairs (CαH and NH), (NH and NH), and (CβH and NH), respectively, and $b_{\beta\beta}$ is for the pair of CβH of two S-bridged residues; shaded bands are for strong NOEs and lines for weak NOEs; broken lines are for strong sequential NOEs for the pair of CαH$_2$ of Gly^8 and CδH$_2$ of Pro^9.

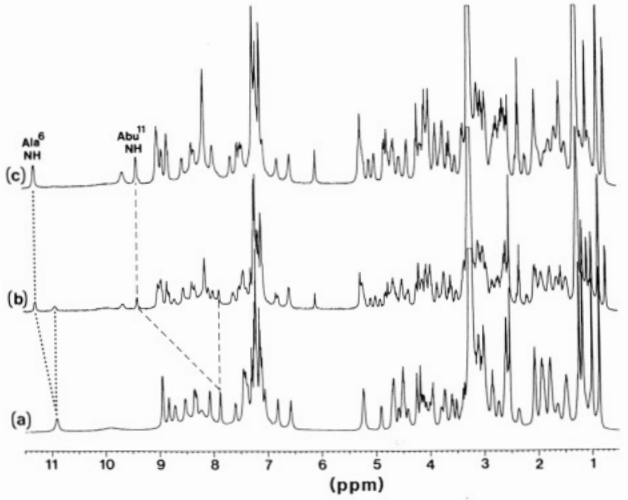

FIGURE 2: 500-MHz ^{1}H NMR spectra of Ro09-0198 in $(CH_3)_2SO$-d_6 solution at 45 °C in the absence (a) and in the presence of 0.5 mol/mol of peptide (b) and 1 mol/mol of peptide (c) of C12-LPE. Peptide concentration is about 5 mM.

As for the interaction of peptide antibiotics with phospholipids, however, amino acid residues involved in lipid binding or conformational changes associated with lipid binding have not been elucidated in detail. In the present study, we carried out proton NMR analyses of the interaction of Ro09-0198 with lysophosphatidylethanolamine in perdeuterated dimethyl sulfoxide solution. We succeeded in the determination of amino acid residues involved in the binding with phosphatidylethanolamine and found an overall conformation change of the peptide upon binding with the phospholipid.

Materials and Methods

Immunopotentiator Ro09-0198 was a generous gift from Dr. Takemoto of Nippon Roche Research Center. 1-Dodecanoyl-*sn*-glycerophosphoethanolamine (C12-LPE)[1] was purchased from Avanti Polar-Lipids Inc. (Pelham, AL). Proton NMR spectra were recorded on Bruker AM-400 and AM-500 spectrometers. Chemical shifts were measured relative to the methyl proton resonance of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. For NMR measurements, the peptide was dissolved in $(CH_3)_2SO$-d_6, evaporated to remove residual water, and redissolved in $(CH_3)_2SO$-d_6 to a final concentration of about 5 mM. When the same peptide was dissolved in water, the pH of the solution was about 5.

We chose $(CH_3)_2SO$-d_6 as the solvent in the present study because of the following reasons. (1) The peptide is readily soluble in $(CH_3)_2SO$-d_6 while it is hardly soluble in methanol or in acetone. (2) C–H proton resonances of the peptide in aqueous solution are significantly shifted with concentration (and temperature), indicating self-aggregation, but not in $(CH_3)_2SO$-d_6 solution (data not shown). (3) In $(CH_3)_2SO$-d_6 solution, peptide N–H proton resonances are clearly observed, allowing unambiguous resonance assignments. Results obtained with this fully dispersed system will provide a basis for elucidating the interaction of the peptide with the phospholipid membranes in more biological aqueous system.

Two-dimensional double quantum filtered COSY (DQF-COSY) (Rance et al., 1983), HOHAHA (Bax & Davis, 1985), and NOESY (Jeener et al., 1979) experiments were performed in the phase-sensitive mode with a time proportional phase increment (Bodenhausen et al., 1984). The mixing time was set to 62 ms for HOHAHA experiments and to 300 ms for NOESY experiments. The data size in the time domain was 512 points in t_1 and 2K points in t_2. For each t_1 value, 64 transients were accumulated. Prior to Fourier transformation, time domain data were multiplied by a squared cosine bell for DQF-COSY and by a Gaussian function for HOHAHA and NOESY. The same window function was applied for t_1 and t_2. The "zero-filling" method was employed to give a frequency domain data size of 1 K × 4K, but symmetrization was not applied. All the NMR measurements were made at 45 °C. The peptide solution (either in the presence or absence of C12-LPE) was stable, and no spectral change was observed over several months.

Results

Resonance Assignments of Free Peptide in $(CH_3)_2SO$-d_6 Solution. While our NMR analysis was in progress, Kessler et al. (1987, 1988) reported on the determination of the chemical structure of Ro09-0198 by analyses of 600-MHz NMR spectra in $(CH_3)_2SO$-d_6 solution. They assigned proton resonances to individual residues by DQF-COSY and elucidated connectivities by NOESY and DQF-relayed NOESY. Our results are mostly in agreement with theirs except that, in 500-MHz spectra, we could not distinguish the spin system of the lysinoalanine residue from that of phenylalanine residues (to be described later). However, we could clearly distinguish between two geminal protons for CδH$_2$ of Pro^9, CβH$_2$ of Phe^{12}, and CβH$_2$ of Lys^{19}. In addition, we confirmed the resonance assignment of β-hydroxyaspartic acid in position 15 by a specific broadening due to the paramagnetic Gd^{3+} ion (data not shown). Our experimental conditions were set appropriate for studying the effect of the interaction of C12-LPE with the peptide, so that the proton chemical shifts of the free peptide observed in the present study are listed in Table I.

Titration of Ro09-0198 with C12-LPE. Upon addition of C12-LPE to a $(CH_3)_2SO$-d_6 solution of Ro09-0198, a new set of resonances due to the peptide–LPE complex appeared while the intensities of proton resonances of the free peptide were

[1] Abbreviations: Abu(S)Ala, β-methyllanthionine residue [NHCH(CO)CH(CH$_3$)SCH$_2$CH(CO)NH]; Ala(S)Ala, lanthionine residue [NHCH(CO)CH$_2$SCH$_2$CH(CO)NH]; C12-LPE, 1-dodecanoyl-*sn*-glycerophosphoethanolamine; DQF-COSY, double quantum filtered correlation spectroscopy; DQF-relayed NOESY, double quantum filtered, relayed nuclear Overhauser enhancement spectroscopy; HO-Asp, β-hydroxyaspartic acid residue [NHCH(CO)CH(OH)COOH]; HOHAHA, homonuclear Hartmann–Hahn correlation spectroscopy; Lys(N)Ala, lysinoalanine residue [NHCH(CO)(CH$_2$)$_4$NHCH$_2$CH(CO)NH]; NOESY, nuclear Overhauser enhancement spectroscopy.

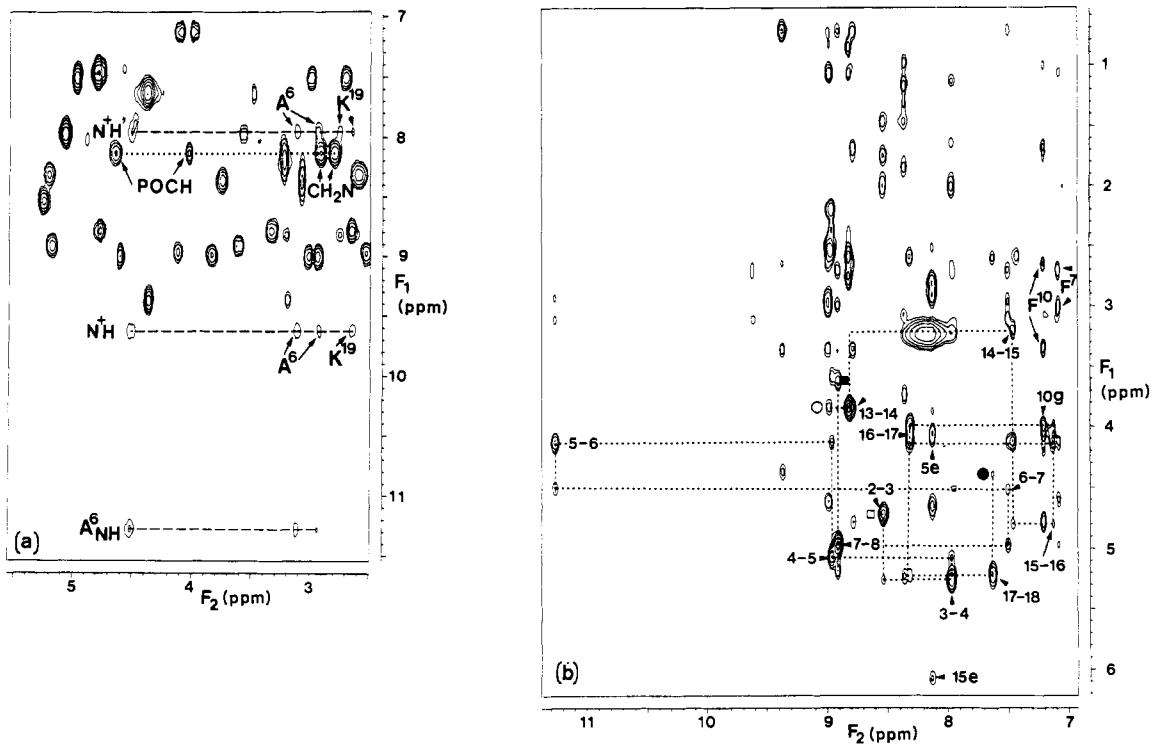

FIGURE 3: (a) 500-MHz HOHAHA spectrum (N–H/CαH, CβH region) of the 1:1 complex of Ro09-0198 and C12-LPE in $(CH_3)_2SO$-d_6 solution at 45 °C. The mixing time is 62 ms. The cross-peaks due to the NH_2^+ protons of Lys^{19}-NH_2^+-Ala^6 are connected by thick broken lines, where the locations of Ala(N)^6 CβH and Lys^{19} CϵH resonances are marked with A^6 and K^{19}, respectively. The cross-peaks due to the amide proton of Ala(N)^6 are connected by a thin broken line. The cross-peaks due to the NH_3^+ protons of C12-LPE are connected by a dotted line. (b) 500-MHz NOESY spectrum (aliphatic/amide region) of the 1:1 complex of Ro09-0198 and C12-LPE in $(CH_3)_2SO$-d_6 solution at 45 °C. Sequential NOE peaks for pairs of CαH of one residue and N–H of the succeeding residue are marked with residue number pairs and are connected, by broken lines, with intraresidue NOE cross-peaks of the succeeding residue. A connectivity starts from the interresidue NOE for the pair of Arg^2 and Gln^3 (marked with open square) and ends with the intraresidue NOE of Gly^8 (closed square). Another connectivity starts from the intraresidue NOE of Val^{13} (open circle) and ends with the intraresidue NOE of Abu(S)^{18} (closed circle). The cross-peaks due to the sequential NOE for pairs (Pro^9, Phe^{10}), (Phe^{10}, Abu(S)^{11}), and (Abu(S)^{11}, Phe^{12}) are observed with a lower contour level (data not shown). The intraresidue NOE for the pairs of Cβ,β' protons and C2,6 protons of Phe^7 and Phe^{10} are marked with F^7 and F^{10}, respectively. The intermolecular NOE cross-peaks for proton pairs (OH of HO-Asp^{15} and NH_3^+ of C12-LPE), (CαH of Ala(S)^5 and NH_3 of C12-LPE), and (C2,6H of Phe^{10} and glycerol C2H of C12-LPE) are marked with 15e, 5e, and 10g, respectively.

decreased (Figure 2). For example, changes in amide proton resonance peaks of the free state (a) and the bound state (c) are marked for Ala(N)^6 (with dotted lines) and Abu(S)^{11} (with broken lines). The exchange rate of the peptide molecule between the free state and the LPE-bound state was found to be slow since changes in chemical shift or line width of free peptide resonances were not observed either.

Resonance Assignment of LPE-Bound Peptide in $(CH_3)_2SO$-d_6 Solution. Assignments of proton resonances of the peptide in the presence of C12-LPE were carried out as in the absence of C12-LPE. First, peptide proton resonances were classified into amino acid residue types by tracing *J* connectivities in the DQF-COSY spectrum with the aid of relay peaks in the HOHAHA spectrum. Thus, the proton resonances of Arg^2, Pro^9, Abu(S)^{11}, Val^{13}, HO-Asp^{15}, Abu(S)^{18}, and Lys^{19} were readily identified. The resonances of Asn^{17} was assigned by the characteristic NOESY peaks for the pair of the Cβ protons and the carboxamide protons. Note that each of the two nonequivalent NH_2^+ protons of lysinoalanine-19/6 were observed in the HOHAHA spectrum, exhibiting cross-peaks with the Cϵ protons of Lys^{19} and with the Cβ protons of Ala^6 (thick broken lines in Figure 3a). This enabled an unambiguous resonance assignment of lysinoalanine in the presence of C12-LPE, whereas in the absence of C12-LPE the NH_2^+ proton resonances were not observed probably because of proton exchange with trace water.

Sequential Assignment of Ala(S)^1–Pro^9. Further, sequential assignments of spin systems were made by observation of NOESY cross-peaks for the pairs of the Cβ proton of the *i*th residue and the amide proton of the (*i* + 1)th residue (Wüthrich, 1986). The characteristic spin system of Pro^9 was readily identified in the DQF-COSY spectrum. From this residue, main-chain protons were traced toward the amino terminus. Observation of the NOESY cross-peaks for the pairs of the two Cδ protons of Pro^9 and the two Cα protons of Gly^8 (GP in Figure 4b) allowed the assignment of the proton resonances of Gly^8. The spin system of Phe^7 was found from the NOESY cross-peak for the amide proton of Gly^8 and the Cα proton of Phe^7 (7–8 in Figure 3b). The connectivity of Phe^7 and Ala(N)^6 was confirmed by the observation of the cross-peak for the Cα proton of Ala(N)^6 and the amide proton of Phe^7 (6–7 in Figure 3b). The proton resonances of Ala(S)^5 were identified from the cross-peak for the pair of the Cα proton of Ala(S)^5 and the amide proton of Ala(N)^6 (5–6 in Figure 3b). In a similar manner, the main-chain proton resonances of the sequence Arg^2-Gln^3-Ala(S)^4-Ala(S)^5 were assigned (Figure 3b). The amide proton resonance of Arg^2 was not observed, because of a fast exchange rate of the proton in the lipid-bound state. Nevertheless, the proton resonances of Ala(S)^1 were identified by the observation of the NOESY cross-peaks for the pair of the two Cβ protons of Ala(S)^1 and the Cβ proton of Abu(S)^{18} (B1 in Figure 4b).

Sequential Assignment of Phe^{10}–Phe^{12}. The proton resonances of Phe^{10} were identified by the NOESY cross-peak for the pair of the Cα proton of Pro^9 and the amide proton of Phe^{10}. In a similar manner, the spin connectivity of Phe^{10}-Abu(S)^{11}-Phe^{12} was traced, which was confirmed by observation of NOESY cross-peaks for the pairs of amide protons

Table I: Chemical Shifts (ppm) of Ro09-0198 in the Absence (Roman) and in the Presence (Italic) of C12-LPE and Chemical Shifts of LPE in the Absence (Roman) and in the Presence (Italic) of Ro09-0198 in $(CH_3)_2SO$-d_6 Solution at 45 °C

amino acid	N–H	CαH	CβH	others
Ala(S)[1]	*a*	4.17	3.72, 3.06	
	a	*4.19*	*3.70, 3.05*	
Arg[2]	9.92	4.70	1.77, 1.47	1.64, 1.48 (CγH)
	a	*4.72*	*1.74, 1.46*	*1.59, 1.45*
				3.09, 3.09 (CδH)
				3.08, 3.08
				8.46 (NδH)
				8.38
Gln[3]	8.51	5.22	1.92, 1.92	2.06, 2.06 (CγH)
	8.53	*5.26*	*1.95, 1.95*	*2.01, 2.01*
				7.03, 6.56 (NH_2)
				7.04, 6.55
Ala(S)[4]	8.06	4.90	3.58, 2.53	
	7.96	*5.07*	*3.59, 2.36*	
Ala(S)[5]	8.93	4.47	2.53, 2.35	
	8.96	*4.12*	*2.54, 2.19*	
Ala(N)[6]	10.90	4.65	3.03, 2.96	
	11.27	*4.51*	*3.11, 2.94*	
Phe[7]	8.70	4.51	3.14, 2.84	*b* (C2,6H)
	7.50	*4.97*	*3.00, 2.70*	*7.08*
Gly[8]	7.43	4.08, 4.08		
	8.91	*5.19, 3.61*		
Pro[9]		3.95	1.90, 1.72	2.05, 1.89 (CγH)
		4.05	*1.78, 1.01*	*1.66, 1.66*
				3.72, 3.42 (CδH)
				3.84, 3.35
Phe[10]	8.92	3.97	2.99, 2.99	7.09 (C2,6H)
	8.78	*4.77*	*3.35, 2.65*	*7.21*
Abu(S)[11]	7.86	4.49	3.25	1.16 (methyl)
	9.37	*4.37*	*3.20*	*0.73*
Phe[12]	8.21	4.66	3.36, 3.00	
	9.00	*4.61*	*3.01, 2.94*	
Val[13]	7.38	4.23	1.78	0.98, 0.85 (methyl)
	8.98	*3.84*	*2.53*	*1.07, 0.86*
Ala(S)[14]	8.81	3.30	2.84, 2.59	
	8.81	*3.20*	*2.75, 2.61*	
HO-Asp[15]	7.42	4.58	4.24	3.60 (OH)
	7.46	*4.81*	*4.75*	*6.08*
Gly[16]	7.35	4.14, 4.00		
	7.12	*4.09, 4.00*		
Asn[17]	8.31	5.22	2.58, 2.58	7.39, 6.80 (NH_2)
	8.31	*5.21*	*2.59, 2.59*	*7.42, 6.78*
Abu(S)[18]	7.58	4.40	3.50	1.23 (methyl)
	7.63	*4.38*	*3.49*	*1.23*
Lys[19]	8.34	3.79	1.77, 1.26	1.22, 1.03 (CγH)
	8.42	*3.67*	*1.83, 1.13*	*0.98, 0.98*
				1.47, 1.13 (Cδ)
				1.63, 1.16
				2.73, 2.62 (CεH)
				2.74, 2.64
				a, a (NεH)
				9.61, 7.97
LPE	2.30 (Cα)	4.01, 3.96 ($C1H_2O$)		3.69, 3.69 ($C3H_2OP$)
	2.32	*4.19, 4.06*		*3.70, 3.70*
	1.54 (Cβ)	3.77 (C2H)		2.98, 2.98 (CH_2N)
	1.55	*3.97*		*2.91, 2.80*
	1.27 (Cγ)	5.69 (C2OH)		3.87, 3.87 ($POCH_2$)
	1.27	*5.25*		*4.65, 4.03*
	0.87 (methyl)			8.30 (NH_3)
	0.87			*8.13*

[a] Not observed. [b] Not determined.

of two consecutive residues (data not shown).

Sequential Assignment of Val[13]–Lys[19]. The proton resonances of Val[13] were readily assigned in the DQF-COSY spectrum from observation of spin coupling of two methyl resonances with one Cβ proton. The connectivity of Val[13]-Ala(S)[14]-HO-Asp[15]-Gly[16]-Asn[17]-Abu(S)[18] was determined from the NOESY cross-peaks for the pair of the Cα proton of the *i*th residue and the amide proton of the $(i + 1)$th residue (Figure 3b). The connectivity of Abu(S)[18] to Lys[19] was

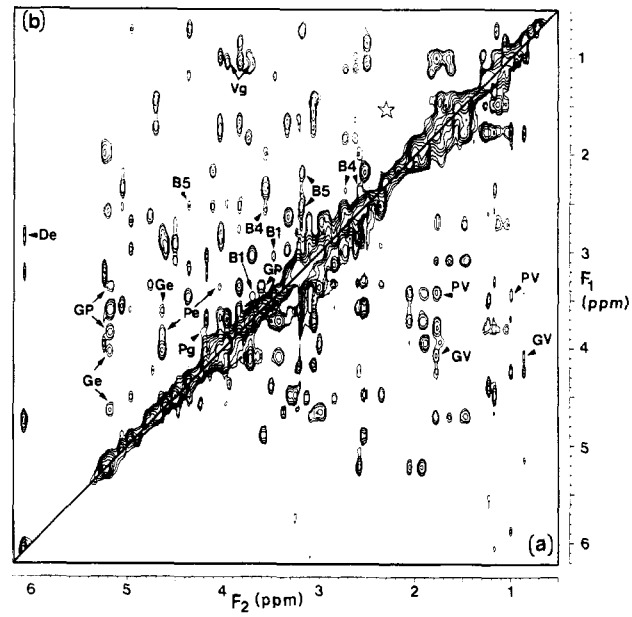

FIGURE 4: 500-MHz NOESY spectra (aliphatic/aliphatic region) of Ro09-0198 in $(CH_3)_2SO$-d_6 solution at 45 °C in the absence (a) and in the presence (b) of C12-LPE. The mixing time is 300 ms. (a) Interresidue NOE peaks for the pairs of Gly[8] and Val[13] and of Pro[9] and Val[13] are marked with GV and PV, respectively. (b) Sequential NOE peaks for the pairs of Gly[8] Cα,α′ and Pro[9] Cδ,δ′ are marked with GP. For S-bridged residues, Ala(S)[1]-Abu(S)[18], Ala(S)[4]-Ala(S)[14], and Ala(S)[5]-Abu(S)[11], NOE peaks are marked with B1, B4, and B5, respectively. Intermolecular NOE peaks are marked as follows: Gly[8] Cα,α′H and ethanolamine $POCH_2$ (Ge); Pro[9] CδH and glycerol C1H (Pg); Pro[9] Cδ,δ′H and ethanolamine $POCH_2$ (Pe); Val[13] CH_3 and glycerol C2H and $C3H_2$ (Vg); HO-Asp[15] OH and ethanolamine CH_2N (De). An open star is for the missing NOE for the pair of αCH_2 and βCH_2 of the dodecanoyl chain of C12-LPE.

identified from the cross-peak for the amide protons of these residues (data not shown).

Finally, these assignments were confirmed by the observation of NOESY cross-peaks for the proton pairs of S-bridged residues, namely, the pairs of the two Cβ protons of Ala(S)[4] and the two Cβ protons of Ala(S)[14] and the pairs of the two Cβ protons of Ala(S)[5] and the Cβ proton of Abu(S)[11] (B4 and B5, respectively, in Figure 4b). The spin connectivities in Ro09-0198 in the presence of C12-LPE are schematically summarized in Figure 1b. The connectivity of Phe[12] and Val[13] was not confirmed here because of overlap of the amide proton resonances of these residues, although such a connectivity was established in the absence of C12-LPE (data not shown). The proton chemical shifts of Ro09-0198 in the presence of C12-LPE are listed in Table I.

Resonance Assignment of C12-LPE. Proton chemical shifts of C12-LPE in $(CH_3)_2SO$-d_6 solution in the absence and in the presence of Ro09-0198 are listed in Table I. In the absence of the peptide, the proton resonances of the glycerol moiety were distinguished from those of the ethanolamine moiety by spin-decoupling experiments. The methylene proton resonances of glycerol $C3H_2OP$ and ethanolamine $POCH_2$ were identified from the spin coupling of 6 Hz with the phosphorus nucleus. In the presence of Ro09-0198, proton resonances of the ethanolamine moiety were readily assigned from the spin coupling of CH_2N and NH_3^+; HOHAHA cross-peaks were observed for the NH_3^+ proton and four nonequivalent CH_2CH_2 protons (dotted line in Figure 3a). The proton resonances of the glycerol moiety (nonequivalent $C1H_2$ and C2H and equivalent $C3H_2$ protons) were recognized from the HOHAHA cross-peaks for the C2OH proton, which was confirmed by the DQF-COSY analysis (data not shown). The proton

resonances of the dodecanoyl chain were readily assigned both in the absence (by spin decoupling) and in the presence (by DQF-COSY) of the peptide (data not shown).

Intermolecular NOE of Ro09-0198 and C12-LPE. The NH_3^+ protons of C12-LPE show rather strong NOESY cross-peaks with the Cβ proton and the hydroxyl proton of HO-Asp[15] (15e in Figure 3b), indicating formation of an ion pair of the ethanolamine moiety of C12-LPE and the carboxylate group of HO-Asp[15]. These NH_3^+ protons also show cross-peaks with the amide protons of Ala(N)[6] and Phe[7] (data not shown) and the Cα proton of Ala(S)[5] (5e in Figure 3b). The two nonequivalent CH_2N protons of C12-LPE also show NOESY peaks with the hydroxyl proton of HO-Asp[15] (De in Figure 4b). The two nonequivalent protons of $POCH_2$ of C12-LPE show cross-peaks with the two Cα protons of Gly[8] and with the two Cδ protons of Pro[9] (Ge and Pe, respectively, in Figure 4b). As for the glycerol moiety, the C2H and two equivalent $C3H_2$ protons show cross-peaks with the lower field methyl resonance of Val[13] (Vg in Figure 4b). The C2H proton also shows a cross-peak with the aromatic C2,6H protons of Phe[10] (10g in Figure 3b). The lower field resonance, rather than the higher field resonance, of the C1-methylene group of the glycerol moiety shows a cross-peak with the lower field resonance of the Cδ methylene group of Pro[9] of Ro09-0198 (Pg in Figure 4b).

As for the dodecanoyl chain, however, even intrachain NOESY cross-peaks such as that for the pair of the Cα and Cβ protons were not observed (Figure 4b, the position marked by an open five-pointed star), indicating high mobility of the acyl chain and the absence of an interaction between the dodecanoyl chain of C12-LPE and the peptide.

Discussion

The proton chemical shifts of Ro09-0198 in the free state and in the LPE-bound state are listed in comparison in Table I. The amino acid residues in this peptide can be readily classified into two groups; the proton resonances of the first group are significantly shifted upon binding with LPE but not those of the second group.

Central Part [Ala(N)[6]–HO-Asp[15]]. The amino acid residues of the first group are found in the central part of the sequence [Ala(N)[6]-Phe[7]-Gly[8]-Pro[9]-Phe[10]-Abu(S)[11]-Phe[12]-Val[13]-Ala-(S)[14]-HO-Asp[15]]. Among them, the significant shifts of the side-chain proton resonances of HO-Asp[15] (+0.51 ppm for CβH and +2.48 ppm for O–H) upon binding with C12-LPE are obviously due to an ion pair formation of this side-chain group with the ethanolamine group of the lipid. The formation of this ion pair is confirmed by observation of NOESY cross-peaks for the pair of these groups. Significant shifts of proton resonances upon binding with C12-LPE are also found for residues Ala(N)[6], Phe[7], Gly[8], Pro[9], Phe[12], and Val[13], which suggest direct interactions of these residues with the lipid head group. Such interactions are supported by observation of NOESY cross-peaks, which further indicates concomitant conformational changes. In fact, for the sequence from Ala(N)[6] to Ala(S)[14], a conformation change is clearly found from the differences in NOE involving Gly[8], Pro[9], and Val[13], which will be discussed below.

In the absence of C12-LPE, long-range NOE cross-peaks are observed for the proton pairs [Gly[8] CαH_2, Val[13] CβH], [Gly[8] CαH_2, Val[13] CH_3 (higher field)], [Pro[9] Cδ'H (higher field), Val[13] CβH], and [Pro[9] Cδ'H, Val[13] CH_3 (lower field)] (GV and PV in Figure 4a), indicating a direct contact of Gly[8]-Pro[9] with Val[13] (Figure 5a). In a preliminary molecular modeling study, the ring formed by the sequence from Ala(S)[4] to Ala(S)[14] and a thioether bridge can be folded about another

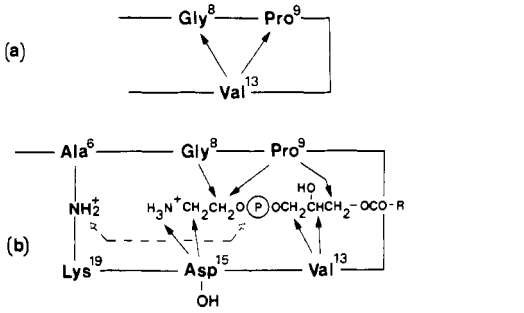

FIGURE 5: Intramolecular and intermolecular interactions (schematic) (a) in free Ro09-0198 and (b) in the complex of Ro09-0198 and C12-LPE. Intramolecular interactions are found for the pair of Gly[8] and Val[13] and of Pro[9] and Val[13] in the free peptide (a). These intramolecular interactions are replaced with intermolecular interactions: (glycerol) $C1H_2$ with Pro[9], C2H with Val[13], and $C3H_2$ with Val[13]; (ethanolamine) $POCH_2$ with Gly[8] and Pro[9], CH_2N with HO-Asp[15], and NH_3^+ with HO-Asp[15]. The dodecanoyl chain of C12-LPE is not involved in the interaction with the peptide.

bridge, Ala(S)[5]–Abu(S)[11], thus bringing Val[13] in contact with Gly[8] and Pro[9]. This folded structure is probably stabilized by formation of interstrand hydrogen bonds such as between Gly[8] NH and Val[13] CO and between Pro[9] CO and Phe[12] NH.

By contrast, in the presence of C12-LPE, NOE cross-peaks noted above are all missing and replaced by *intermolecular* NOE peaks for pairs such as of Gly[8] CαH_2 and ethanolamine $POCH_2$, of Val[13] CH_3 and glycerol C2H, and of Val[13] CH_3 and glycerol $C3H_2$. These results indicate that the glycerophosphoethanolamine moiety of C12-LPE wedges into the Ro09-0198 molecule by breaking an intramolecular contact between Gly[8]-Pro[9] and Val[13] (Figure 5b). This is supported by all the intermolecular NOE cross-peaks for the pairs of peptide protons and lipid protons as described under Intermolecular NOE of Ro09-0198 and C12-LPE. Note that such an intermolecular contact and a concomitant conformation change can only be elucidated by analyses of NOE cross-peaks rather than CD spectra.

While the protonated ethanolamine moiety of C12-LPE is clearly found to interact with the carboxylate group of HO-Asp[15], identification of a putative positively charged group that interacts with the negatively charged phosphate group of C12-LPE is not straightforward. None of the three positively charged residues [Ala(S)[1], Arg[2], and Lys[19]-N-Ala[6]] shows a large chemical shift change upon binding with C12-LPE. However, formation of an ion pair between the secondary ammonium group of Lys[19]-NH_2^+-Ala[6] and the phosphate group of C12-LPE is suggested by the observation of the secondary ammonium proton resonance (not observed in the free state), which may be ascribed to a slowed exchange of ammonium protons with residual water. In addition, from a preliminary molecular modeling study, a positively charged group that possibly interacts with the phosphate group of LPE is found to be restricted to the secondary ammonium group of Lys[19]-N-Ala[6].

Terminal Parts. The amino acid residues of the second group include Ala(S)[1]–Ala(S)[5] in the amino terminus and Gly[16]–Lys[19] in the carboxyl terminus. The proton resonances of these residues are not shifted significantly upon binding with C12-LPE, and intermolecular NOESY cross-peaks are not observed either. However, these residues in the amino and carboxyl termini are presumably involved in stabilization of the active conformation of Ro09-0198.

Conformation and Action of Lipid-Bound Peptides. In a series of conformation studies on the phospholipids interaction of α-mating factor, luteinizing hormone releasing hormone, and analogue peptides, we found that the physiological ac-

tivities of these peptides are related to the conformation in the membrane-bound state rather than to the conformation in the free state (Higashijima et al., 1983, 1988; Wakamatsu et al., 1983, 1986a,b,c, 1987; Tanaka et al., 1986). On the other hand, a hydrophobic labeling study on adrenocorticotropic hormone and analogue peptides indicated that the biological activities of those peptides are related to the affinity to phospholipid membrane (Gysin & Schwyzer, 1983). Probably, physiologically active peptides from aqueous media are trapped by the membrane lipids, take some ordered conformation, and then move within the membrane finally to bind with their specific receptors. Such schemes have been postulated for the function of physiologically active peptides (Epand, 1983; Gysin & Schwyzer, 1983; Higashijima et al., 1983; Deber & Behnam, 1984; Kaiser & Kézdy, 1987; Schwyzer, 1987; Higashijima et al., 1988).

The peptide antibiotic Ro09-0198 possibly binds with a receptor in a similar way. Note that the amount of the specific receptor phosphatidylethanolamine is rather limited in the outer leaflet of the cell membrane (Rothman & Lenard, 1977; Venien & Le Grimellec, 1988). Nevertheless, Ro09-0198 has a slight affinity for phosphatidylcholine (data not shown), the most abundant phospholipid in the outer leaflet of the cell membrane. Such binding, if weak, with phosphatidylcholine will facilitate the specific binding of this peptide with phosphatidylethanolamine through concentration of the peptide around the membrane.

Interaction of Ro09-0198 with Phospholipid Membrane. Even with a small molecular size, Ro09-0198 has been found to strictly recognize phosphatidylethanolamine (Choung et al., 1988a,b). In fact in the present study, a specific multisite binding of Ro09-0198 with the glycerophosphoethanolamine moiety was elucidated; Gly^8, Pro^9, Val^{13}, and HO-Asp^{15} are in contact with CH_2CH_2 of the ethanolamine moiety and with CH_2CHCH_2 of the glycerol moiety (Figure 5). The γ-carboxylate group of HO-Asp^{15} and the secondary ammonium group of lysinoalanine-19/6 are in contact with the postively charged amino group and the negatively charged phosphate group of the phosphoethanolamine moiety. Specific binding to the membrane phospholipid facilitates activities of the peptide such as hemolysis or permeability enhancement through the membrane. Ro09-0198 is an elaborately designed peptide with not only cognitive but also "active" membrane-damaging functions. Compare the molecular weight of this peptide (2000) with that of immunoglobulin G (1.5×10^5). Thus, the interaction of this antibiotic peptide with (lyso)-phosphatidylethanolamine will provide a suitable model for analyzing intermolecular recognition and for designing an artificial "minienzyme".

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References

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355–360.

Bodenhausen, G., Kogler, H., & Ernst, R. R. (1984) *J. Magn. Reson. 58*, 370–388.

Choung, S.-Y., Kobayashi, T., Inoue, J., Takemoto, K., Ishitsuka, H., & Inoue, K. (1988a) *Biochim. Biophys. Acta 940*, 171–179.

Choung, S.-Y., Kobayashi, T., Takemoto, K., Ishitsuka, H., & Inoue, K. (1988b) *Biochim. Biophys. Acta 940*, 180–187.

Deber, C. M., & Behnam, B. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 61–65.

Epand, R. M. (1983) *Trends Biochem. Sci. 8*, 205–207.

Gysin, B., & Schwyzer, R. (1983) *FEBS Lett. 158*, 12–16.

Higashijima, T., Fujimura, K., Masui, Y., Sakakibara, S., & Miyazawa, T. (1983) *FEBS Lett. 159*, 229–232.

Higashijima, T., Wakamatsu, K., Milon, A., Okada, A., & Miyazawa, T. (1988) in *Peptide Chemistry 1987* (Shiba, T., & Sakakibara, S., Eds.) pp 679–688, Protein Research Foundation, Osaka, Japan.

Jeener, J., Meier, B. H., Bachman, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.

Kaizer, E. T., & Kézdy, F. J. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 561–581.

Kessler, H., Steuernagel, S., Gillessen, D., & Kamiyama, T. (1987) *Helv. Chim. Acta 70*, 726–741.

Kessler, H., Steuernagel, S., Will, M., Jung, G., Kellner, R., Gillessen, D., & Kamiyama, T. (1988) *Helv. Chim. Acta 71*, 1924–1929.

Navaro, J., Chabot, J., Sherrill, K., Aneja, R., Zahler, S. A., & Racker, E. (1985) *Biochemistry 24*, 4645–4650.

Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479–485.

Rothman, J. E., & Lenard, J. (1977) *Science 195*, 743–753.

Schwyzer, R. (1987) in *Peptides 1986* (Theodoropoulos, D., Ed.) pp 7–23, Walter de Gruyter, Berlin and New York.

Shotwell, O. L., Stodola, F. H., Michael, W. R., Lindenfelser, L. A., Dworschack, R. G., & Pridham, T. G. (1958) *J. Am. Chem. Soc. 80*, 3912–3915.

Takemoto, K. (1979) British Patent Application 7911395.

Tanaka, H., Osakada, F., Kubo, K., Mukai, H., Muramatsu, R., Munekata, E., Wakamatsu, K., Miyazawa, T., Ohashi, S., & Shiraki, M. (1986) in *Peptide Chemistry 1985* (Kiso, Y., Ed.) pp 311–316, Protein Research Foundation, Osaka, Japan.

Venien, C., & Le Grimellec, C. (1988) *Biochim. Biophys. Acta 942*, 159–168.

Wakamatsu, K., Higashijima, T., Fujino, M., Nakajima, T., & Miyazawa, T. (1983) *FEBS Lett. 162*, 123–126.

Wakamatsu, K., Higashijima, T., & Miyazawa, T. (1986a) in *Natural Products and Biological Activities* (Imura, H., Goto, T., Murachi, T., & Nakajima, T., Eds.) pp 237–244, University of Tokyo Press, Tokyo.

Wakamatsu, K., Okada, A., Suzuki, M., Higashijima, T., Masui, Y., Sakakibara, S., & Miyazawa, T. (1986b) *Eur. J. Biochem. 154*, 607–615.

Wakamatsu, K., Okada, A., Higashijima, T., & Miyazawa, T. (1986c) *Biopolymers 25*, S193–S200.

Wakamatsu, K., Okada, A., Miyazawa, T., Masui, Y., Sakakibara, S., & Higashijima, T. (1987) *Eur. J. Biochem. 163*, 331–338.

Wakamiya, T., Fukase, K., Naruse, N., Konishi, M., & Shiba, T. (1988) *Tetrahedron Lett. 29*, 4771–4772.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley-Interscience, New York.