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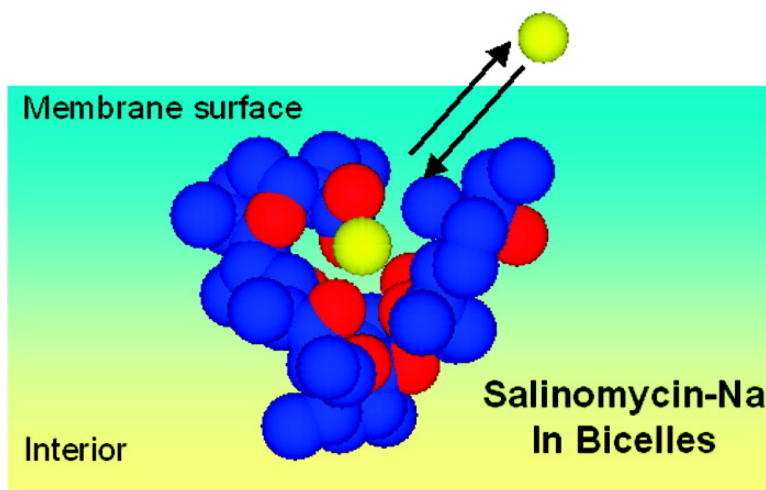

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Conformation and Location of Membrane-Bound Salinomycin−Sodium Complex Deduced from NMR in Isotropic Bicelles

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Abstract: An ionophore antibiotic salinomycin was studied in a membrane environment consisting of isotropic bicelles, a better model for biological membranes than micelles, and its conformation and topological orientation in bicelles was determined. 2D NMR measurements and restrained conformational search revealed that salinomycin−sodium salt in bicelles adopts an open conformation in which the orientation of the E-ring is significantly different from that in crystal and solution structures. This conformational alteration breaks an intramolecular hydrogen bond between 28-OH and 1-O, dislocates the ether oxygen of the E-ring from a coordinated position to the sodium ion observed in the crystal, and consequently weakens the complexation between salinomycin and the sodium ion. Paramagnetic relaxation experiments using doxyl-phospholipids reveal that salinomycin is embedded shallowly in bicelles, with both terminals being closer to the water interface and the olefin portion facing the bicelle interior. Measurements of intermolecular NOEs between salinomycin and phospholipids further supported this orientation. Weaker complexation with sodium ion and positional preference in the membrane polar region may facilitate the catch-and-release of metal ions at the polar/nonpolar interface of bilayers. On the basis of these findings, a model for salinomycin-assisted transport of metal ions across biological membranes is proposed.

Introduction

Ionophore polyether antibiotics,[1] such as monensin, narasin, salinomycin, and lasalocid, which mediate the transport of various metal ions across biological membranes, have been the subjects of intensive studies because of their physiological and pharmacological significance. However, so far, very little is known about the biologically active conformation of ionophores when they are binding and transporting metal ions. Therefore, determination of the 3D structure of ionophore−metal complexes in membrane environments is a prerequisite for understanding the mechanism of ion transport through biological membranes by the antibiotics.

To mimic the membrane environment, sodium dodecyl sulfate (SDS) or other surfactant micellar media have frequently been used,[2−4] and indeed monensin was subjected to conformational analysis in micelles.[5] However, use of micelles as a membrane model is controversial because of the small radius of curvature and the lack of planar bilayer structures. Recently, it has been shown that phospholipid bicelles provide a more natural membrane environment because of the presence of planar lipid bilayer portions.[6,7] Bicelles generally consist of long- and short-chain phospholipids, and their size and shape are controlled by the ratio (q) of the long-chain phospholipids to the short ones. At higher q values (>2.5), bicelles adopt a magnetically aligned lamellar bilayer morphology, presumably like sliced Swiss cheese.[8−13] Recently, magnetically oriented bicelles are being used as model membranes in solid-state NMR studies on membrane-associated peptides and proteins.[14] In contrast, with a decrease in q values, the aggregate becomes disk-shaped,[15−20] where the planar bilayer region formed by the long-chain lipid

(1) For a review, see: *Polyether Antibiotics*; Westley, J. W., Ed.; Marcel Dekker: New York 1982; Vols 1−2.
(2) Fernandez, C.; Wüthrich, K. *FEBS Lett.* **2003**, *555*, 144−150.
(3) Mascioni, A.; Porcelli, F.; Ilangovan, U.; Ramamoorthy, A.; Veglia, G. *Biopolymers* **2003**, *69*, 29−41.
(4) Porcelli, F.; Buck, B.; Lee, D.-K.; Hallock, K. J.; Ramamoorthy, A.; Veglia, G. *J. Biol. Chem.* **2004**, *279*, 45815−45823.
(5) Mercurio, E.; Pellegrini1, M.; Mierke, D. F. *Biopolymers* **1997**, *42*, 759−769.
(6) Sanders, C. R.; Hare, B. J.; Howard, K. P.; Prestegard, J. H. *Prog. Nucl. Magn. Reson. Spectrosc.* **1994**, *26*, 421−444.
(7) Chou, J. J.; Kaufman, J. D.; Stahl, S. J.; Wingfield, P. T.; Bax, A. *J. Am. Chem. Soc.* **2002**, *124*, 2450−2451.
(8) Gaemers, S.; Bax, A. *J. Am. Chem. Soc.* **2001**, *123*, 12343−12352.
(9) Nieh, M. P.; Glinka, C. J.; Krueger, S.; Prosser, R. S.; Katsaras, J. *Langmuir* **2001**, *17*, 2629− 2638.
(10) Sanders, C. R.; Landis, G. C. *Biochemistry* **1995**, *34*, 4030−4040.
(11) Howard, K. P.; Prestegard, J. H. *J. Am. Chem. Soc.* **1996**, *118*, 3345−3353.
(12) Losonczi, J. A.; Prestegard, J. H. *Biochemistry* **1998**, *37*, 706−716.
(13) Sanders, C. R.; Landis, G. C. *J. Am. Chem. Soc.* **1994**, *116*, 6470−6471.
(14) Dürr, U. H. N.; Yamamoto, K.; Im, S.-C.; Waskell, L.; Ramamoorthy, A. *J. Am. Chem. Soc.* **2007**, *129*, 6670−6671.
(15) Lin, T. L.; Liu, C. C.; Roberts, M. F.; Chen, S. H. *J. Phys. Chem.* **1991**, *95*, 6020−6027.
(16) Vold, R. R.; Prosser, R. S. *J. Magn. Reson., Ser. B* **1996**, *113*, 267−271.
(17) Glover, K. J.; Whiles, J. A.; Wu, G. H.; Yu, N. J.; Deems, R.; Struppe, J. O.; Stark, R. E.; Komives, E. A.; Vold, R. R. *Biophys. J.* **2001**, *81*, 2163−2171.
(18) Luchette, P. A.; Vetman, T. N.; Prosser, R. S.; Hancock, R. E. W.; Nieh, M. P.; Glinka, C. J.; Krueger, S.; Katsaras, J. *Biochim. Biophys. Acta* **2001**, *1513*, 83−94.
(19) van Dam, L.; Karlsson, G.; Edwards, K. *Biochim. Biophys. Acta* **2004**, *1664*, 241−256.
(20) Andersson, A.; Mäler, L. *Langmuir* **2006**, *22*, 2447−2449.

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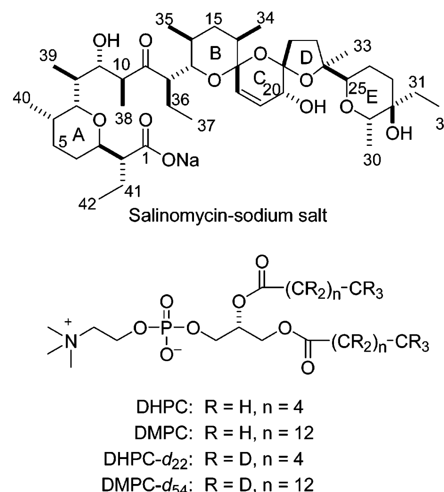
is surrounded by a rim consisting of the short-chain lipid. Bicelles with $q < 1$ are known to have fast-tumbling and isotropic properties in aqueous solutions with retaining the discoidal shape.[17−20] Isotropic bicelles permit high-resolution NMR measurements of membrane-bound molecules and, therefore, have been used for structural determinations of small membrane peptides.[21−28] Recently, we have used isotropic bicelles for determining the membrane-bound structure of erythromycin A, a widely prescribed macrolide antibiotic, and demonstrated the general utility of isotropic bicelle media in determining the conformation and location of membrane-associated nonpeptidic molecules.[29]

Salinomycin,[30] a representative member of ionophore polyether antibiotics, mediates the transport of various metal ions, especially sodium and potassium, across biological membranes.[31] Because of its wide spectrum against Gram-positive bacteria and coccid, salinomycin is commonly used as an effective veterinary drug. The crystal structures and solution structures of salinomycin−sodium salt in $CDCl_3$ and DMSO-d_6 have been published.[32,33] These studies showed that the conformation of the antibiotic varied slightly depending on the environment, whereas the complexation pattern and the geometry of the coordination sphere of the sodium ion remained largely unaffected.[33] Although these reports provided significant insight into specific requirements of metal binding for this important polyether ionophore, the structure in membrane environments has yet to be determined. Hence, in the present work, we used isotropic bicelles in the structural study of salinomycin in a membrane environment. In addition, the topological orientation of salinomycin within bicelles was also determined by monitoring the radical-induced relaxation of proton signals in the presence of doxyl-phospholipids. On the basis of these results, we will discuss a mechanism for transportation of metal ions across membranes by salinomycin.

Results

Conformations of Salinomycin in Bicelles. In the present study, we used typical isotropic bicelles composed of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) at a ratio of 1:2 ($q = 0.5$). Salinomycin-sodium salt was incorporated into the isotropic bicelles at 5 mol % of total phospholipids. The total lipid concentration was set to 8% W/V in a 100 mM NaCl aqueous solution. The prepared bicelle sample was clear and stable for more than 2 weeks.

Chart 1. Structures of Salinomycin−Sodium Salt, DHPC, DMPC, DHPC-d_{22}, and DMPC-d_{54}



Prior to NMR measurements of salinomycin in isotropic bicelles, to examine whether the bicelle structure is maintained in the presence of salinomycin, we first measured ^{31}P NMR spectra of large oriented bicelles ($q = 3.5$) containing salinomycin at 5 mol % of total phospholipids. In oriented bicelles with the normal perpendicular to the direction of the magnetic field, ^{31}P NMR spectra show two well-resolved resonances: the high-field resonance is attributed to DMPC in the planar section, whereas the downfield resonance is attributed to DHPC on the edges of the disks or covering the Swiss cheese perforations.[34,35] An increase in the magnitude of the isotropic ^{31}P NMR signal generally indicates membrane lysis by a drug, since the lysis results in an increased amount of small fast tumbling phospholipid structures such as mixed micelles. Hence, ^{31}P NMR is useful in probing the effect of a drug on the morphology of bicelles. The resultant ^{31}P spectrum observed for oriented bicelles containing salinomycin was typical of well-aligned bicelles (Figure S1, Supporting Information). Although the ^{31}P signals are slightly broadened in the presence of salinomycin, this result totally shows that salinomycin does not act as a detergent destroying bicelle structure, thus confirming the applicability of the bicellar system to the following structural study of salinomycin.

For measuring the NMR spectra of salinomycin in isotropic bicelles, we used deuterated phospholipids DMPC-d_{54} and DHPC-d_{22}, in which hydrogen atoms in acyl chains are replaced by deuteriums. A series of COSY, TOCSY, and NOESY spectra were utilized to fully assign the proton resonances (Table 1).

The conformation of salinomycin associated with isotropic bicelles was determined based on the NOEs and $^3J_{HH}$ values obtained from the NOESY and DQF-COSY experiments, respectively. NOESY cross-peaks were categorized into three groups with upper distance limits of 3.0, 4.0, and 5.0 Å (Table 1) based on NOE intensities. We also utilized $^3J_{HH}$ values to restrain the dihedral angles in the C2−C3, C7−C10, and C12−C13 bonds of salinomycin (Table 2). The typical large and small values of the vicinal coupling constants, as listed in Table 2, indicate that rotational averaging rarely takes place for these

(21) Lindberg, M.; Biverståhl, H.; Gräslund, A.; Mäler, L. *Eur. J. Biochem.* **2003**, *270*, 3055−3063.
(22) Vold, R. R.; Prosser, S. R.; Deese, A. J. *J. Biomol. NMR* **1997**, *9*, 329−335.
(23) Andersson, A.; Mäler, L. *J. Biomol. NMR* **2002**, *24*, 103−112.
(24) Whiles, J. A.; Brasseur, R.; Glover, K. J.; Melacini, G.; Komives, E. A.; Vold, R. R. *Biophys. J.* **2001**, *80*, 280−293.
(25) Bárány-Wallje, E.; Andersson, A.; Gräslund, A.; Mäler, L. *FEBS Lett.* **2004**, *567*, 265−269.
(26) Marcotte, I.; Separovic, F.; Auger, M.; Gagne, S. M. *Biophys. J.* **2004**, *86*, 1587−1600.
(27) Glover, K. J.; Whiles, J. A.; Wood, M. J.; Melacini, G.; Komives, E. A.; Vold, R. R. *Biochemistry* **2001**, *40*, 13137−13142.
(28) Guo, J.; Pavlopoulos, S.; Tian, X.; Lu, D.; Nikas, S. P.; Yang, D.-P.; Makriyannis, A. *J. Med. Chem.* **2003**, *46*, 4838−4846.
(29) Matsumori, N.; Morooka, A.; Murata, M. *J. Med. Chem.* **2006**, *49*, 3501−3508
(30) Miyazaki, Y.; Shibuya, M.; Sugawara, H.; Kawaguchi, O.; Hirose, C.; Nagatsu, J.; Esumi, S. *J. Antibiot.* **1974**, *28*, 814−821.
(31) Dobler, M. *Ionophores and Their Structures*; John Wiley & Sons: New York, 1981.
(32) Mronga, S.; Mueller, G.; Fischer, J.; Riddell, F. *J. Am. Chem. Soc.* **1993**, *115*, 8414−8420.
(33) Paulus, E. F.; Kurz, M.; Matter, H.; Vértesy, L. *J. Am. Chem. Soc.* **1998**, *120*, 8209−8221.
(34) Marcotte, I.; Dufourc, E. J.; Ouellet, M.; Auger, M. *Biophys. J.* **2003**, *85*, 328−339.
(35) Dvinskikh, S. V.; Dürr, U. H. N.; Yamamoto, K.; Ramamoorthy, A. *J. Am. Chem. Soc.* **2007**, *129*, 794−802.

Table 1. ^{1}H Chemical Shifts and NOE Data of Salinomycin−Sodium Salt in Isotropic Bicelles

position	δ_H	NOEs[a]	position	δ_H	NOEs[a]
2	2.85	5bs, 7s, 41m, 42s	23a	2.21	23bs, 25w, 26am, 26bm, 33m
3	3.93	4as, 4bs, 41m, 42w	23b	1.82	
4a	1.46	4bs	25	3.62	26as, 27aw, 30s, 33s
4b	1.88	40s	26a	1.43	26bs, 27aw
5a	1.43	5bs, 6s	26b	1.71	27as, 27bs
5b	1.88	6s, 39w, 40s	27a	1.6	27bs
6	1.78	7m, 39s, 40s	27b	1.21	
7	3.73	39m, 40s	29	3.86	30s, 31abm
8	1.50	9s, 38s, 39s, 40s	30	1.2	31s, 32w
9	4.07	38s	31ab	1.34	32s
10	2.94	12s, 13w, 38s, 39s	32	0.90	
12	2.7	13s, 14w, 35s, 36aw, 36bm, 38m	33	1.43	
13	3.82	14m, 15abm, 18s, 19w, 35s	34	0.74	
14	1.7	15bw, 35s	35	0.89	
15a	1.61	15bs, 18w	36a	1.86	37s
15b	1.16	18s, 34m	36b	1.56	37s
16	1.71	34s	37	0.89	
18	6.15	19s, 34m	38	0.84	
19	5.97	20m	39	0.75	
20	4.04	34m	40	0.93	
22a	2.35	22bs	41ab	1.35	42s
22b	1.99	23abm	42	0.93	

[a] NOE connectivities are listed once from the proton having the lower number to the higher number. The intensity of NOEs is represented as strong (s), medium (m), or weak (w).

Table 2. $^3J_{HH}$ Values of Salinomycin−Sodium Salt in Isotropic Bicelles[a]

coupled protons	H-2/H-3	H-7/H-8	H-8/H-9	H-9/H-10	H-12/H-13
$^3J_{HH}$ (Hz)	12	10	1	11	1

[a] Data were extracted from the DQF-COSY spectrum (see Supporting Information).

acyclic portions. The total numbers of interproton distance and dihedral restraints derived from the NMR data were 78 and 5, respectively. Upon calculation, we applied a dielectric constant value of 20 because, as described later, a salinomycin molecule mostly interacts with the polar region of bilayers whose dielectric constant was estimated to be larger than 20.[36,37] Under these conditions, 3000 random conformers were generated by using a systematic Monte Carlo conformation search algorithm,[38] and each conformer was subjected to energy minimization by the conjugate gradient method using MMFF force field.[39]

Figure 1 shows an ensemble of the 20 lowest-energy conformations of the salinomycin−sodium complex in isotropic bicelles with energies less than 3.4 kJ/ mol. Although the whole molecule appears well-converged, the structure in bicelles has a major alteration in the C24−C25 bond; i.e., the orientation of the E-ring is significantly different from that in the crystal state or in organic solvents. In the bicelle structure, the dihedral angle of C23−C24−C25−25O takes a gauche conformation, while an anti conformation is dominant in the crystal and

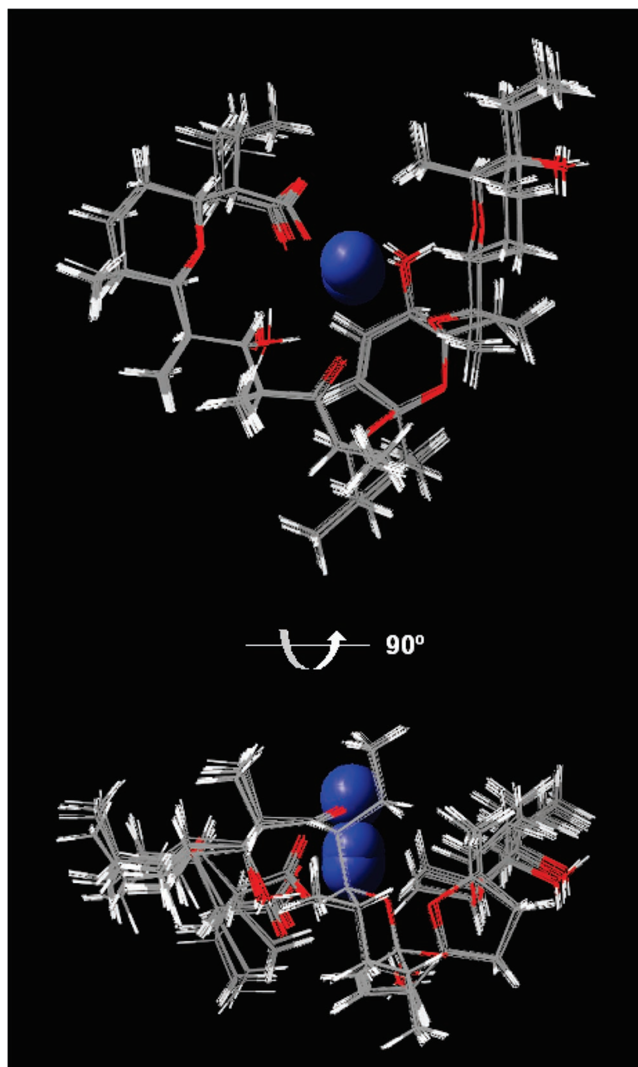


Figure 1. Ensemble of the 20 lowest-energy conformations of salinomycin−sodium complex of energies less than 3.4 kJ/mol in isotropic bicelles ($q = 0.5$). The calculation was performed with the systematic Monte Carlo method constrained by NOE and $^3J_{HH}$ data.

solution structures.[33] Because the rotation of the C24−C25 bond was not restricted by $^3J_{HH}$ values in the calculation, the conformational difference is stemmed from distance constraints based on NOEs (details will be discussed later).

Another interesting point in Figure 1 is that the sodium ion is not fixed and has significant disorder along one direction, although the salinomycin molecular frame has only minor conformational fluctuations. When the calculation was performed without applying the NMR constraints and the dielectric constant, the position of the ion was completely fixed, and the conformation obtained was very similar to the crystal structure (data not shown). Therefore, the positional disorder of the ion is a characteristic of the current calculation. This positional fluctuation of the sodium ion indicates that the potential energy curve is shallow with respect to the position of the sodium ion, and therefore, seems to be related to the dynamic process of catch-and-release of the sodium ion. We will discuss this point later.

Location of Salinomycin in Bicelles. Paramagnetic ions (i.e., Mn^{2+}, Gd^{3+}, and Dy^{3+}) and nitroxyl spin-labels have long been used for determining the depth of bound molecules in micelles

(36) Ashcroft, R. G.; Coster, H. G. L.; Smith, J. R. *Biochim. Biophys. Acta, Biomembranes* **1981**, *643*, 191−204.
(37) Raudino, A.; Mauzerall, D. *Biophys. J.* **1986**, *50*, 441−449.
(38) Goodman, J. M.; Still, W. C. *J. Comput. Chem.* **1991**, *12*, 1110−1117.
(39) Halgren, T. A. *J. Comput. Chem.* **1996**, *17*, 490−512, 520−552, 553−586, 587−615, 616−641.

and lipid vesicles. Paramagnetic agents enhance the relaxation of NMR nuclei in their vicinity. The relaxation induced by Mn^{2+} has frequently been used to estimate molecular segments that are exposed to the aqueous exterior of membranes.[21,40,41] Similarly, 5-doxyl and 12-doxyl phospholipids were used to detect NMR nuclei present in the hydrophobic interior of membranes.[21] Both were used in this study to determine which protons were exposed to the water−lipid interface and which were confined to the hydrophobic bicelle interior. The paramagnetic contribution to spin−lattice relaxation is best represented by T_{1M}

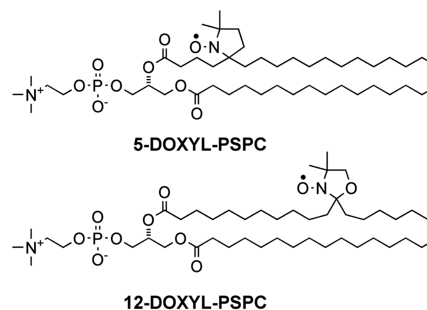
$$\frac{1}{T_{1M}} = \frac{1}{T_{1P}} - \frac{1}{T_1^{\,0}}$$

where T_1^0 is the spin−lattice relaxation time in the absence of paramagnetic agents, and T_{1P} is the time in their presence.[42] Paramagnetic relaxation time T_{1M} has explicit r^6 distance dependency, which makes it possible to semiquantitatively measure the depth of membrane-bound entities. We have already shown that these paramagnetic probes are properly located in isotropic bicelles.[29]

We first measured T_{1M} values of salinomycin protons in bicelles containing Mn^{2+} (see Supporting Information), though only a limited number of T_{1M} values were available because of overlapping signals on 1D-NMR. Unexpectedly, Mn^{2+} ions strongly influenced H-2, H-10, H-12, H-18, and H-19, which are supposed to be situated inside the ion-coordinating cavity of salinomycin. Because salinomycin is known to interact with divalent cations such as Ba^{2+} and Ca^{2+},[43] the unusual paramagnetic effects of Mn^{2+} on the drug are thought to be due to a complex formation with Mn^{2+} in place of Na^+.

Next, we measured the T_{1M} values of salinomycin protons in bicelles containing 1-palmitoyl-2-stearoyl-5 (or 12)-doxyl-*sn*-glycero-3-phosphocholine (5- or 12-doxyl PSPC) (Figure 2). Although all of the protons are more or less influenced by the doxyl-PSPCs, H-18 and H-19 are the most significantly affected (Figure 2), suggesting the deeper immersion of these protons into the bicelle interior. In contrast, H-2 and H-30 located at both terminals of the molecule are less affected by the nitroxyl radicals, indicating that the molecular termini are more distant from the bicelle interior, i.e., closer to the membrane surface. More importantly, both 5- and 12-doxyl-PSPCs show similar paramagnetic relaxation profiles to salinomycin protons (Figure 2). This indicates that the nitroxyl radicals of both doxyl-PSPCs have similar orientations toward the drug with respect to the membrane normal. Because the radical of 5-doxyl-PSPC is attached to the C5 of a phospholipid acyl chain, similar relaxation effects of both doxyl-PSPCs on salinomycin suggest that the entire molecule of salinomycin is localized predominantly above the C5 position of phospholipid acyl chains.

The position of salinomycin was further examined by intermolecular NOEs between salinomycin and phospholipid, which were measured effectively using the Gradient-enhanced nuclear Overhauser Effect SpectroscopY (GOESY) method.[44]



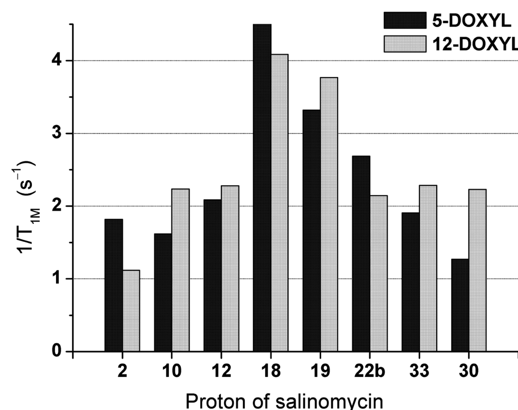


Figure 2. Structures of 5- and 12-doxyl-PSPCs (top) and their effects on proton relaxation times of salinomycin−sodium salt in isotropic bicelles (bottom). The panel depicts the inverse of T_{1M} in the presence of 1 mM of doxyl-PSPC.

For this experiment, we used bicelles composed of DHPC-d_{22} and DMPC to observe proton signals of DMPC. Selective irradiation at H-2 of salinomycin gave rise to a distinct NOE enhancement at choline protons of phospholipid (Figure 3). This NOE suggests that the carboxylate group of salinomycin resides predominantly at the water−lipid interface. Another GOESY experiment with irradiation at H-18 of salinomycin gave an NOE at methylene protons of the phospholipid acyl chain (Figure 4a), indicating that H-18 of salinomycin is closer to the bicelle interior. These NOE data are consistent with the results of the aforementioned paramagnetic relaxation experiments.

Next, we examined whether salinomycin binds to the flat membrane-like area on the bicelle. For this purpose, we used the intermolecular NOE between methylene protons of the phospholipid and H-18 of salinomycin (Figure 4). Isotropic bicelles were composed of either DHPC-d_{22}/DMPC (Figure 4a) or DHPC/DMPC-d_{54} (Figure 4b). Disappearance of the NOE at the methylene protons in DHPC/ DMPC-d_{54} bicelles (Figure 4b) indicates that the intermolecular NOE is not due to the methylene protons of DHPC but mainly due to those of DMPC. This shows that salinomycin is predominantly distributed in the DMPC-rich flat domain of the bicelle structure.

Discussion

This paper reports the conformation of salinomycin in the membrane-mimetic environment using isotropic bicelles for the first time. The conformation obtained has a distinctive difference in E-ring orientation from that in the crystal state and organic

(40) Tuzi, S.; Hasegawa, J.; Kawaminami, R.; Naito, A.; Saito, H. *Biophys. J.* **2001**, *81*, 425−434.
(41) Buffy, J. J.; Hong, T.; Yamaguchi, S.; Waring, A. J.; Lehrer, R. I.; Hong, M. *Biophys. J.* **2003**, *85*, 2363−2373.
(42) Villalaín, J. *Eur. J. Biochem.* **1996**, *241*, 583−593.
(43) Suzuki, K.; Tohda, K.; Aruga, H.; Matsuzoe, M.; Inoue, H.; Shirai, T. *Anal. Chem.* **1988**, *60*, 1714−1721.
(44) Stonehouse, J.; Adell, P.; Keeler, J.; Shaka, A. J. *J. Am. Chem. Soc.* **1994**, *116*, 6037−6038.

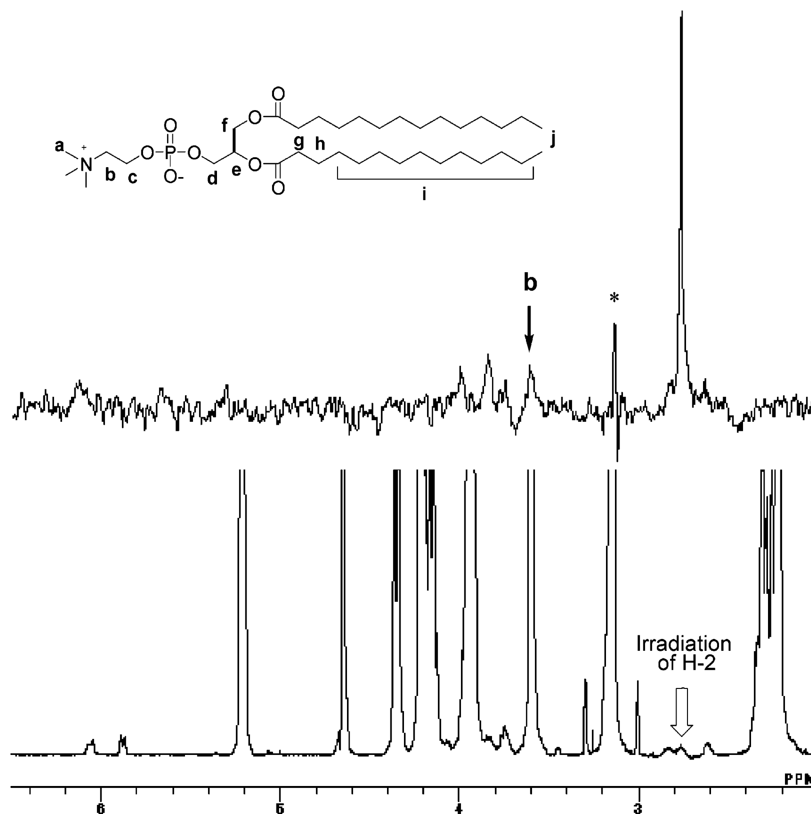


Figure 3. 1D-GOESY spectrum of isotropic bicelles (DHPC-d_{22}/DMPC 2:1) containing salinomycin (top), and conventional ^{1}H spectrum (bottom). In the GOESY spectrum, a selective pulse was applied to H-2 of salinomycin, and the mixing time was set to 1000 ms. A weak intermolecular NOE was observed at b protons of phospholipids. This NOE suggests that the carboxylate of salinomycin resides in the polar region of phospholipids. Other enhancements are assigned to intramolecular NOEs. The signal denoted by "*" is probably an artifact.

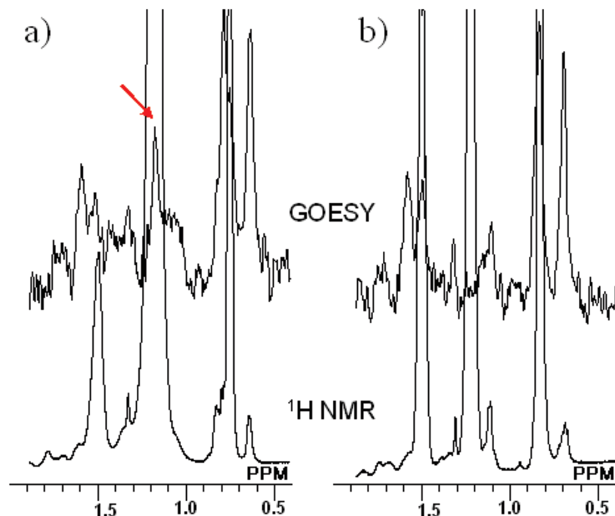


Figure 4. NOE enhancement at methylene protons of bicelle phospholipids measured by GOESY experiments irradiating H-18 of salinomycin. Isotropic bicelles were composed of DHPC-d_{22}/DMPC (a), or DHPC/DMPC-d_{54} (b). Mixing time was set to 1000 ms. Disappearance of the NOE in DHPC/DMPC-d_{54} bicelles (b) indicates the preferential distribution of salinomycin in the DMPC-rich planar region of the bicelle structure.

solvents (Figure 1).[32,33] As described above, because the conformation of C24−C25 is not constrained by coupling constants, the orientational change in the E-ring is attributed to different NOE patterns. To confirm the conformational alteration around the C24−C25 bond, we refer here to the difference in NOE features and chemical shifts in bicelles and organic solvents. In organic solvents ($CDCl_3$ and DMSO-d_6), strong NOEs were reported between H-23 and H-25,[32,33] indicating a gauche orientation of the dihedral angle of C23−C24−C25−H-25, whereas the corresponding NOE in bicelles is very weakly observed, suggesting an anti conformation. Similarly, NOEs between H-23 and H-26 were not reported in organic solvents,[32,33] whereas the NOE was relatively intense in bicelles. ^{1}H chemical shifts were also different around the bond in bicelles and in $CDCl_3$; the chemical shift differences exceed 0.2 ppm in the C23−C29 region, whereas those in other portions almost agree within 0.1 ppm. These facts support the orientational change in the E-ring revealed by the restrained conformational analysis. Indeed, Anteunis et al.[45] proposed the torsion of C24−C25 to be one of the major hinges when salinomycin changes from a closed to an open conformation and vice versa. In this context, the structure in bicelles (Figure 1) may represent one of the open conformations of salinomycin. On the other hand, conformations in the other portions do not differ significantly between the current work and the earlier report on the crystal and solution structures.[33] In effect, coupling constants in acyclic portions of salinomycin are largely unchanged irrespective of solvent system.[32,33] Similarly, NOE patterns in bicelles agree with those in organic solvents except for protons in the E-ring and its neighbor.[32,33] These facts are also consistent with the notion that only the E-ring orientation is significantly changed in the bicelle system.

Here, the question arises as to why the E-ring orientation varies only in the bicelle environment. As revealed by doxyl-PSPCs and GOESY experiments, salinomycin is embedded

(45) Anteunis, M. J. O.; Rodios, N. A. *Bull. Soc. Chim. Belg.* **1981**, *90*, 715−735.

relatively shallowly in the bicelles; in particular, the terminal E-ring is most likely to reside in a water accessible region. The crystal and solution structures have hydrophobic molecular surfaces because most of the oxygenated functional groups are directed inside to coordinate with the sodium ion; therefore the structures are unstable in polar surroundings. In contrast, the bicelle structure has free oxygenated functionalities at the E-ring such as 28-OH and 25-O that can form hydrogen bonds with water and polar heads of phospholipids. Therefore, it seems reasonable to consider that the orientational change in the E-ring enhances molecular hydrophilicity, and consequently adjusts the molecule to more polar regions in the membrane.

As a result of the orientational change in the E-ring, salinomycin cannot form an intramolecular hydrogen bond between 28-OH and 1-O, which plays a key role in forming closed conformations as reported in the crystal state and in organic solvents.[33] In addition, the change in the E-ring prevents its ether oxygen from coordinating with the sodium ion. The resultant weak complexation of salinomycin with the sodium ion would promote the association/dissociation process of the ion; in contrast, there is little room for the exchange of the cation in the crystal state or in $CDCl_3$ because of the tight complexation.[32] The positional disorder of the sodium ion shown in Figure 1 also indicates the weaker complexation of the ion with salinomycin and the resultant shallow potential energy curve with respect to the position of the sodium ion. Combined with the result that salinomycin is located predominantly at the water–lipid interface, it can be assumed reasonably that the positional disorder of the sodium ion represents the dynamic process of capture and release of the ion at the membrane surface. In other words, the conformation of salinomycin observed in bicelles would reproduce the structure around the membrane surface where metal ions are exchanging. If this is the case, then the association/dissociation of the ion should take place along the vertical direction of the cavity formed by salinomycin, as demonstrated in Figure 1. Since salinomycin was shown to reside in the bilayer portion (not in the rim) of the bicelles, it is probable that the structure and behavior of salinomycin observed here substantially reproduce those in biological membranes.

The most likely mechanism for the ion transport by salinomycin is the association/dissociation process of the ion at the membrane surface and the subsequent diffusion process across the membrane.[46] As already stated, the association/dissociation process at the membrane surface is observed predominantly in the bicelle system, which probably suggests that, as an intermediate, the complex at the membrane surface is thermodynamically more stable than the complex in the diffusion state. Because the previously reported crystal and solution structures seem to mimic those in hydrophobic environments, the use of the bicelle system allowed us, for the first time, to observe the drug's structure that is in the process of association/dissociation of the ion at the water–lipid interface.

On the basis of current findings and previous reports, we propose a model of ion transport across biological membranes promoted by salinomycin (Figure 5). At the membrane surface, both termini of the molecule are closer to the water interface and the olefin portion facing the bicelle interior as determined by the paramagnetic doxyl-PSPCs and intermolecular NOEs.

(46) Riddell, F. G. *Chirality* **2002**, *14*, 121–125.

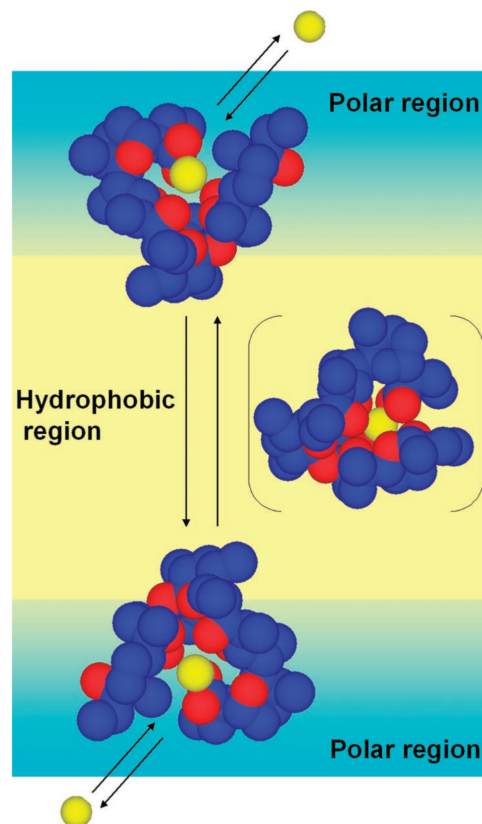


Figure 5. Model representing ion transport across biological lipid bilayers by salinomycin. In the polar region of lipid bilayers, the drug takes an open conformation that facilitates the association/dissociation of metal ions. While diffusing into the hydrophobic region, the drug adopts a closed conformation by rotating the C24–C25 bond as shown in parentheses. The figure in parentheses is taken from the crystal structure.[33]

While diffusing across the nonpolar membrane interior, the molecule takes a closed conformation, as reported in the crystal state or in organic solvents,[33] by rotating the C24–C25 bond. Upon progression from the open state at the bicelle surface, where the sodium ion is weakly bound, to a closed conformation, where the sodium ion is more strongly bound, the sodium ion seems to move from one ligand sphere in the more open conformer environment to an altered ligand sphere in the closed conformer along a shallow potential well. Once closed, the molecular surface is hydrophobic and the metal ion is completely segregated from nonpolar lipid acyl chains, which facilitates diffusion across the membrane.

Conclusion

In this study, we have successfully determined the structure and location of salinomycin bound to a membrane consisting of an isotropic bicelle system. Because salinomycin was shown to reside in the bilayer portion (not in the rim) of the bicelles, it is reasonable to assume that the structure and orientation of salinomycin obtained in this work substantially reproduce those in biological membranes. On the basis of current findings and previous reports, we have deduced the mechanism underlying ion transport across membranes by salinomycin; the drug resides predominantly in the polar region with an open conformation and undergoes the association/dissociation of metal ions, whereas the molecule adopts a closed conformation by rotating the C24–C25 bond for it to be suitable for moving in the hydrophobic membrane interior. This model sheds light on the

structure and dynamics of the important ionophore antibiotic salinomycin, and consequently provides a valuable insight into the mechanism of ion transport for other ionophore antibiotics. This study also clearly shows the general utility of isotropic bicelles for detailed conformational and orientational studies of membrane-associated nonpeptidic drugs.

Experimental Section

Materials. Salinomycin-sodium salt was purchased from Sigma-Aldrich and recrystallizd from acetonitrile-water. Non-labeled and deuterated phospholipids, dimyristoyl-*sn*-glycerophosphatidylcholine (DMPC), DMPC-d_{54}, dihexanoyl-*sn*-glycerophosphatidylcholine (DHPC), DHPC-d_{22}, and the spin-labeled phospholipids 1-palmitoyl-2-stearoyl-*sn*-glycero-5-doxyl-3-phosphatidylcholine (5-doxyl-PSPC) and 1-palmitoyl-2-stearoyl-*sn*-glycero-12- doxyl-3-phosphatidylcholine (12-doxyl-PSPC) were purchased from Avanti Polar Lipids (Alabaster, AL). $MnCl_2$ and NaCl were purchased from Nacalai tesque (Kyoto, Japan), and D_2O from Euriso-Top.

2D NMR Measurements. For two-dimensional NMR measurements, DMPC-d_{54} (5.92 mg, 8.08 μmol), DHPC-d_{22} (7.68 mg, 16.20 μmol), and salinomycin−sodium salt (0.94 mg, 1.21 μmol) were dissolved in 3 mL of $CHCl_3$. The solution was evaporated to a thin film in a flask and dried in vacuo for more than 8 h. To the flask was added 170 μL of 100 mM NaCl in D_2O to give a solution with a total lipid concentration of 8% (w/v). The resultant mixture was occasionally vortexed and incubated at room temperature for 1 h. The obtained clear solution was transferred to a Shigemi 5 mm NMR tube (Shigemi Inc., Tokyo, Japan). The final concentration of salinomycin was 15 mM.

NMR spectra were recorded on JEOL GSX 500 (500 MHz) and JEOL Lambda 500 (500 MHz) spectrometers. All 2D measurements were carried out at 37 °C with a repetition time of 1 s. DQF-COSY, NOESY, and TOCSY spectra were recorded in phase-sensitive mode using the States method, and FG-COSY was in absolute mode. The data points were 1K (F_2) by 512 (F_1) for NOESY and TOCSY, 1K (F_2) by 256 (F_1) for FG-COSY, and 8K (F_2) by 256 (F_1) for DQF-COSY. The spectral width in both dimensions was typically 4500 Hz. The data were apodized with sin-bell window function for FG-COSY and with shifted square sine-bell functions for phase-sensitive 2D spectra. The mixing times of NOESY and TOCSY were 150 and 80 ms, respectively. DANTE water presaturation was applied to DQF-COSY and TOCSY. In all of the two-dimensional spectra, chemical shifts were referenced to the solvent chemical shift (HOD at 4.65 ppm.).

Conformation Analysis. All of the interproton-distance restraints were derived from the two-dimensional NOESY experiments with a mixing time of 150 ms. Seventy-eight distance restraints were divided into three categories according to the NOESY cross-peak intensities. Upper bounds were fixed at 3.0, 4.0, and 5.0 Å for strong, medium, and weak correlations, respectively. Lower bounds of all distance constraints were fixed at 1.8 Å. For stereospecifically assigned diastereotopic methylene protons, the interproton distances were applied to each proton according to the NOE peak intensity. A total of 5 H−C−C−H dihedral angles obtained from $^3J_{HH}$ coupling constants were used as restraints with a ±40° allowance. Conformations were calculated using MacroModel version 8.6. Initial atomic coordinate was generated based on the crystal structure of salinomycin−sodium complex.[33] To this structure was first applied structure minimization, and then the sampling of the conformational space was performed following the systematic pseudo-Monte Carlo (SPMC) method.[38] Three thousand random conformers were generated by SPMC method, and each conformer was minimized by conjugate gradient method using MMFF force field.[39] A dielectric constant value of 20 was applied during the calculation.

Positioning Study. For Mn^{2+} relaxation experiments, a D_2O solution of $MnCl_2$ was added to the bicelle solution to yield a final concentration of 50 μM $MnCl_2$. For the experiments with spin-labeled phospholipids, either 5-doxyl-PSPC or 12-doxyl-PSPC was added to the $CHCl_3$ solution of the deuterated phospholipids and salinomycin to give a final concentration of 1 mM. The subsequent procedures were the same as the aforementioned bicelle preparation. Spin−lattice relaxation times (T_1) were determined using a standard 180°−δ−90° inversion recovery pulse sequence with 10 δ values between 0.1 and 12 s. Measurements were performed at 37 °C for the samples with and without paramagnetic probes.

GOESY Measurements. The samples used for GOESY measurements were prepared in a similar manner as above. The bicelle was composed of DMPC/DHPC-d_{22} (1:2) or DMPC-d_{54}/DHPC (1:2) with salinomycin−sodium salt at 5 mol % of total phospholipids. The GOESY measurements were performed on JEOL Lambda 500 MHz spectrometer equipped with a 5 mm *z*-gradient probe for pulsed field gradient capability. Spectra were measured at 27 °C with a spectral width of 4500 Hz and 8K data points. The recycle time between scans was 4.2 s. The mixing time was 1000 ms, and the number of repetition was 3000. The selective 90° and 180° pulses had durations of 15 and 30 ms, respectively. The strengths of four gradient pulses with a width of 1 ms were 5, −5, 5, and 10 G/cm in this order.

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Supporting Information Available: NMR spectra of salinomycin-Na in bicelles and a figure showing the effects of Mn^{2+} on proton relaxation times. This material is available free of charge via the Internet at http://pubs.acs.org.

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