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ARTICLE

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Location and orientation of Triclosan in phospholipid model membranes

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Abstract Triclosan is a hydrophobic antibacterial agent used in dermatological preparations and oral hygiene products. Although the molecular mechanism of action of this molecule has been attributed to inhibition of fatty acid biosynthesis, earlier work in our laboratories strongly suggested that the antibacterial action of Triclosan is mediated at least partly through its membranotropic effects. In order to assess its location in phospholipid membranes, high-resolution magic-angle spinning natural abundance ^{13}C NMR of Triclosan embedded within egg yolk lecithin model membranes has been used to obtain ^{13}C spin–lattice relaxation times for both Triclosan and lecithin carbon atoms in the presence of Gd^{3+} ions. The results indicate that Triclosan is localized in the upper region of the phospholipid membrane, its hydroxyl group residing in the vicinity of the C=O/C2 carbon atoms of the acyl chain of the phospholipid, and the rest of the Triclosan molecule is probably aligned in a nearly perpendicular orientation with respect to the phospholipid molecule. Intercalation of Triclosan into bacterial cell membranes likely compromises the functional integrity of those membranes, thereby accounting for at least some of this compound's antibacterial effects.

Keywords Magic-angle spinning NMR · Model membranes · Triclosan

Abbreviations COLOC: correlation by long-range coupling · EYL: egg yolk lecithin · HETCOR: heteronuclear chemical-shift correlation · MAS: magic-angle spinning · MLV: multilamellar vesicles

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Introduction

Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol] has been used for many years as a broad-spectrum antibacterial agent in dermatological preparations such as soaps, deodorants, and cosmetics, and in oral hygiene products such as dentifrices and mouthrinses (Bhargava and Leonard 1996; Jones et al. 2000). Triclosan also has anti-inflammatory activity, possibly due to inhibition of interleukin-induced prostaglandinbiosynthesis (Modéer et al. 1996; Skaare et al. 1997; Mustafa et al. 2000), and it reportedly induces apoptosis in human gingival cells (Zuckerbraun et al. 1998). According to an early study on its mechanism of action, low concentrations of Triclosan interfere with bacterial nutrient uptake, whereas high concentrations induce leakage of intracellular components (Regös et al. 1979). It had been presumed that Triclosan has only a membranotropic mode of action, until McMurry et al. (1998) reported that Triclosan inhibits bacterial enoyl-acyl carrier protein reductase. Inhibition of enoyl-acyl carrier protein by Triclosan has since been confirmed by numerous investigators (Heath et al. 1999, 2000; Levy et al. 1999; Qiu et al. 1999; Roujeinikova et al. 1999; Stewart et al. 1999; Parikh et al. 2000). Several mechanisms for bacterial resistance to Triclosan have been identified, including increased gene expression, target mutation, active efflux from cells, and enzyme inactivation/degradation (Schweizer 2001). Amidst all the attention focused on Triclosan's ability to inhibit fatty acid biosynthesis, the original proposal of Regös and Hitz (1974), that Triclosan causes membrane lesions leading to leakage of intracellular components and cell death, has been largely overlooked; indeed, unlike thymol (Shapiro and Guggenheim 1995), another hydrophobic phenolic disinfectant, Triclosan does not perforate cell membranes. Results obtained by Villalaín

et al. (2001) suggested that Triclosan perturbs the packing and interaction between phospholipids and promotes formation of hexagonal-H_{II} phases at temperatures below the expected $L_\beta \rightarrow L_\alpha$ transition temperatures. Therefore, the mode of action of Triclosan is likely to be at least partly attributable to its specific effects on membrane structure.

The present study is aimed at establishing the location and orientation of molecules of Triclosan inside model multilamellar vesicles (MLVs) composed of egg yolk phosphatidylcholine by the application of high-resolution magic-angle spinning (MAS) ^{13}C NMR spectroscopy (Oldfield et al. 1987; Villalaín 1996, 1997; Auger 2000; Bechinger 2000). This was achieved by determining variations in spin–lattice relaxation times (T_1) of phospholipid and Triclosan carbon atoms in the presence of gadolinium trivalent cation (Gd^{3+}), a paramagnetic agent. Since spin–lattice relaxation times of ^{13}C resonances have an explicit r^{-6} distance dependence on paramagnetic ions, information on relative molecular distances between carbon atoms of EYL and carbon atoms of Triclosan can be deduced. Our results show that Triclosan is located in the vicinity of carbons C1/C4 of the phospholipid molecule and that the molecule is probably aligned nearly perpendicular with respect to the phospholipid molecule.

Materials and methods

Materials

Egg yolk lecithin (EYL) was obtained from Avanti Polar Lipids (Birmingham, Ala., USA), Triclosan was kindly provided by CIBA-Geigy (Basel, Switzerland), and $GdCl_3 \cdot 6H_2O$ was purchased from Sigma-Aldrich (Madrid, Spain). All other reagents used were of analytical grade from Merck (Darmstadt, Germany). The purity of EYL before and after NMR measurements was checked by thin-layer chromatography on silica gel plates (Merck) in chloroform/methanol/water (65:25:5 v/v), where it showed only one spot.

Sample preparation

Samples were prepared as previously described (Villalaín 1996). Briefly, 50 mg of EYL in chloroform and, if required, appropriate amounts of Triclosan in chloroform/methanol 1:1 (v/v) in order to obtain a phospholipid/Triclosan molar ratio of 3:1, were dried under a stream of O_2-free N_2 in the dark, then further dried under high vacuum in the dark for at least 3 h. The samples were dispersed and hydrated in 1 mL of H_2O to form MLVs and kept for 25 min at 35–40 °C, with occasional mixing in order to obtain a uniform suspension. To assure sample homogeneity, lipid dispersions were subjected to three freeze–thaw cycles (−80 °C to 35 °C) with occasional vortexing. When required, MLVs were formed in the presence of 1 mM Gd^{3+}.

Solid-state NMR spectroscopy

MAS ^{13}C NMR spectra were acquired on a Bruker 500 MHz Avance spectrometer (Bruker BioSpin, Rheinstetten, Germany) using a Bruker 4-mm high-resolution HR-MAS probe. The parameters used to obtain the spectra have been described previously (Villalaín 1996). The samples were packed into 4-mm zirconia rotors and placed in the spinning module of the HR-MAS probe; no cross-polarization was used. The spinning speed was 6 kHz, regulated to 3 Hz by a Bruker pneumatic unit. A relaxation delay time of 12 s was used, and typical pulse lengths for ^{13}C were of 5 μs. All experiments were carried out at 298 K. Since long T_1 experiments are particularly sensitive to long-term changes in magnet homogeneity, short and long τ values were alternated by the spectrometer computer. In order to check lipid polymorphism in the presence of Gd^{3+}, ^{31}P NMR was used with the following parameters: a gated broad-band decoupling of 10 W, 25 kHz of spectral width, 32k data points, a 120 s interpulse time, and a 90° radiofrequency pulse. A sample containing EYL and Triclosan at a 3:1 molar ratio in the presence of Gd^{3+} showed a broad asymmetrical signal with a low-frequency peak and a high-frequency shoulder, characteristic of bilayer structures (data not shown).

Results and discussion

We have shown previously that Triclosan has a very high lipid/water partition coefficient, that it embeds into phospholipid membranes and disrupts the packing of the phospholipid molecules, and that it induces the appearance of phospholipid lateral phase separation (Villalaín et al. 2001). However, bacterial growth inhibition by Triclosan does not depend upon loss of intracellular constituents (Villalaín et al. 2001). If biological membranes are a target of Triclosan action, then the location of Triclosan in phospholipid bilayers should be compatible with its ability to perturb phospholipid structure and/or interaction. We sought to address this point using a phospholipid membrane model.

MAS ^{13}C NMR spectra of EYL model membranes, either in the absence or in the presence of Triclosan at a molar ratio of 3:1, yielded well-resolved spectra (Fig. 1). In the MAS ^{13}C NMR spectra of samples containing both EYL and Triclosan, besides the phospholipid resonances, some small resonances belonging to Triclosan were identified and resolved (Fig. 1). Assignment of the different resonances of the phospholipid was made as described by Oldfield et al. (1987), whereas Triclosan resonances were assigned using standard $^{1}H/^{13}C$, heteronuclear chemical-shift correlation (HETCOR), and correlation by long-range coupling (COLOC) experiments. The high resolution of the MAS ^{13}C NMR permitted determination of T_1 values for all carbon atoms of EYL (both neat and admixed with Triclosan) as well as for carbon atoms C1, C2, C1′, and C2′ of Triclosan.

Since the resonance relaxation rate for a given carbon atom is proportional to the number of hydrogens bonded to it (n), the relaxation parameter T_1 was scaled by the number of attached hydrogen atoms. Scaled values of the relaxation time, nT_1, for EYL ^{13}C resonances were plotted as a function of their order in the phospholipid molecule (Fig. 2). The nT_1 values of EYL in the absence of Gd^{3+} were quite similar to previously reported nT_1 values (Villalaín 1996). In the presence of Triclosan and in the absence of Gd^{3+} the nT_1 values of EYL were found to be similar to the nT_1 values for pure EYL (Fig. 2). Comparing the nT_1 values of Triclosan and the nT_1 values of the phospholipid in MLVs containing both

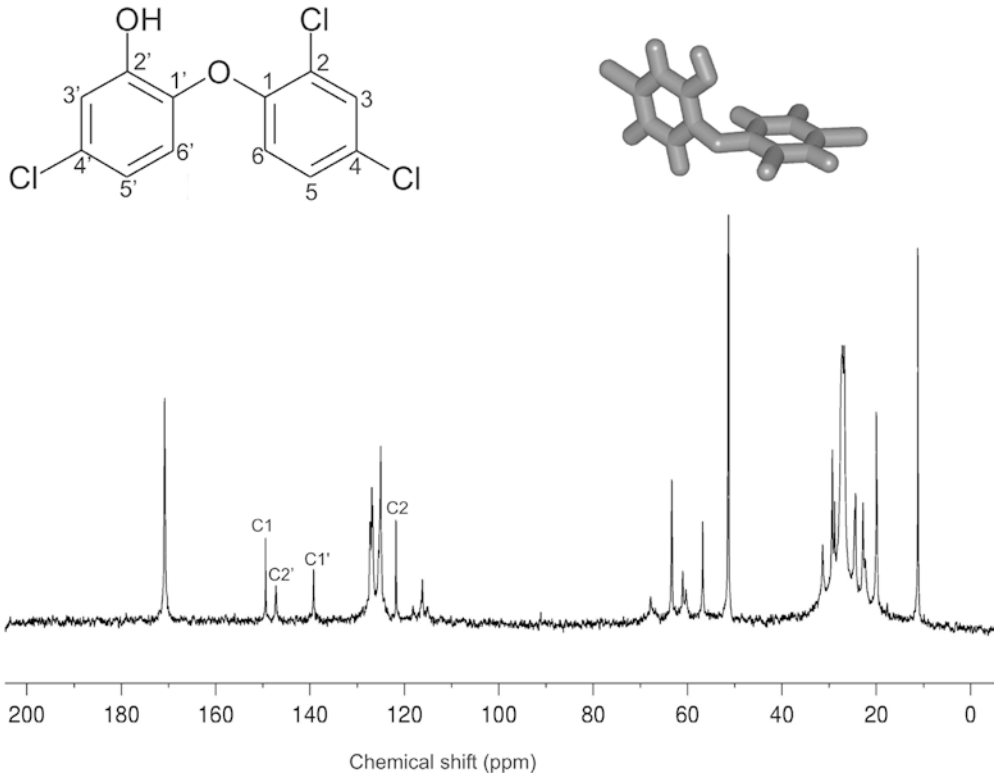

Fig. 1 Proton-decoupled HR-MAS ^{13}C NMR spectrum of fully hydrated bilayers of EYL/Triclosan at a EYL/Triclosan molar ratio of 3:1. The *inserts* show the chemical structure and numbering of Triclosan as well as its three-dimensional structure

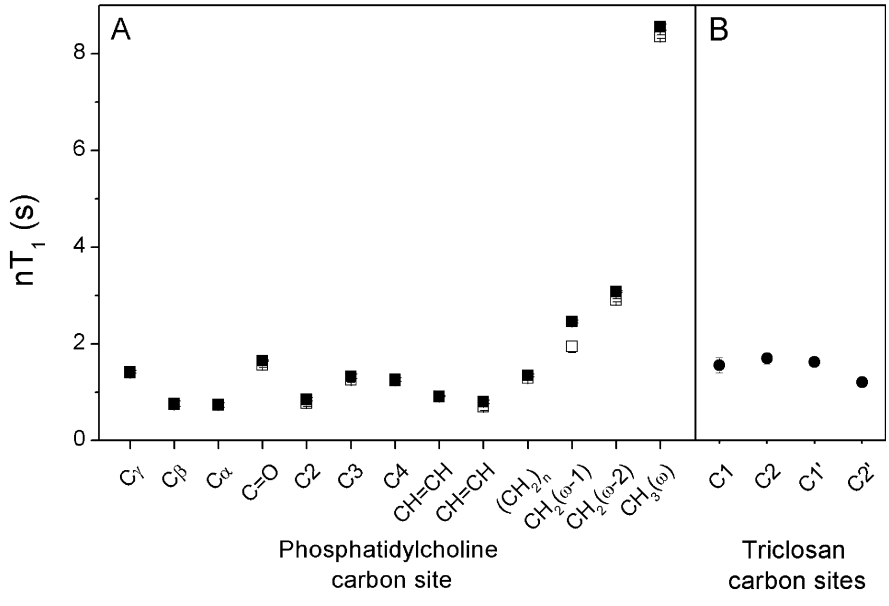

Fig. 2 ^{13}C NMR spin–lattice relaxation times (nT_1 in s) for (**A**) EYL resonances, either in pure form (*open squares*) or in the presence of Triclosan at a EYL/Triclosan molar ratio of 3:1 (*solid squares*) and (**B**) for C1, C2, C1′, and C2′ Triclosan resonances (*solid circles*) as indicated

types of molecules, the nT_1 values of the Triclosan carbons were similar to the nT_1 values of C1–C4 of EYL but different from the nT_1 values of the terminal methylene and methyl carbons; this implies that Triclosan and the upper part of the palisade structure of the membrane have similar molecular motions. Earlier work revealed that the presence of cholesterol (Villalaín 1996) and coenzyme Q (Salgado et al. 1993) in phospholipid membranes did not significantly affect the nT_1 values of EYL, whereas abietic acid (Villalaín 1997), (+)-totarol (Bernabeu et al. 2002), and α-tocopherol (Salgado et al. 1993) noticeably decreased the nT_1 values of the phospholipid hydrocarbon atoms; α-tocopherol also decreased nT_1 values for the headgroup carbons. These differences reflect the different locations within phospholipid membranes of cholesterol and coenzyme Q on the one hand, and abietic acid, (+)-totarol, and, especially, α-tocopherol on the other, the latter compounds residing in the vicinity of the phospholipid polar headgroup. Comparing the nT_1 values of neat EYL

membranes with membranes composed of EYL + Triclosan, it appears that Triclosan molecules likewise are localized in the middle/upper regions of the EYL membrane.

The relative paramagnetic contribution to the spin–lattice relaxation time in the presence of Gd^{3+}, $(T_{1M})^{-1}$, was used to evaluate distances on a molecular scale (Villalaín 1996). The $(T_{1M})^{-1}$ value of the carbonyl carbon of EYL, $(T_{1M}{}^{C=O})^{-1}$, was used to normalize the data, since spin–lattice relaxation times for the different carbons of EYL and Triclosan in samples containing both molecules can be obtained from the same spectrum. In this way, the phospholipid data can be used as an internal control. Values of $[(T_{1M}{}^{X})^{-1}]/[(T_{1M}{}^{C=O})^{-1}]$ for the resonances of EYL in the presence of Triclosan at an EYL/Triclosan molar ratio of 3:1 are shown in Fig. 3. The higher values of $[(T_{1M}{}^{X})^{-1}]/[(T_{1M}{}^{C=O})^{-1}]$ for the carbon atoms of the phospholipid polar headgroup confirmed that the Gd^{3+} ion is effectively bound at the phosphate group of EYL. $[(T_{1M}{}^{X})^{-1}]/[(T_{1M}{}^{C=O})^{-1}]$ values for the carbons of EYL decreased as the distance from the surface of the membrane increased, i.e. the nearer to the middle of the membrane, the smaller was the observed effect (Fig. 3). Therefore, the values of $(T_{1M})^{-1}$ obtained for the different carbons of EYL and Triclosan in the EYL/Triclosan MVLs should enable us to estimate relative distances of the carbons of EYL and Triclosan from the membrane surface where the Gd^{3+} ion is bound.

In spectra of MVLs containing EYL and Triclosan, several Triclosan resonances were clearly resolved, and their $(T_{1M})^{-1}$ values were calculated; this enabled us to compare relative paramagnetic contributions to the spin–lattice relaxation times of the carbons of EYL and Triclosan in the same model membrane system. Assuming an average hydrophobic thickness of 3 nm (Sankaram and Thompson 1992; Ipsen et al. 1990; Nagle 1993; Nezil and Bloom 1992), we prepared a plot of the average distance of the different carbon atoms of EYL (in samples containing EYL/Triclosan at a molar ratio of 3:1) from the membrane surface versus the relative paramagnetic contribution to the spin–lattice relaxation time (Fig. 4). From the $(T_{1M})^{-1}$ values for Triclosan carbon atoms, the relative distances of these carbon atoms from the surface of the membrane could be obtained (Fig. 4). It should be noted that the relationship between $(T_{1M})^{-1}$ and distance is not perfectly linear (Villalaín 1996), possibly due in this case to interactions between Triclosan and EYL (Villalaín et al. 2001).

From the information in Fig. 4 and the fact that the $[(T_{1M}{}^{X})^{-1}]/[(T_{1M}{}^{C=O})^{-1}]$ values for the C2′ carbon of Triclosan (bearing the hydroxyl group) and the C = O group of EYL are very similar, it appears that these two carbon atoms are relatively near to one another. Likewise, C1 and C1′ of Triclosan appear to lie close to and roughly equidistant from C2 and C3 of EYL (with Triclosan C1 possibly slightly closer than Triclosan C1′ to EYL C2), whereas C2 of Triclosan appears to reside in the vicinity of carbons C3 and C4 (closer to C3) of EYL. Since the paramagnetic contribution to the spin–lattice relaxation time is proportional to r^{-6}, where r is the distance between the Gd^{3+} ion and the nucleus in question (Villalaín 1996), the paramagnetic effect decreases very quickly with distance from the paramagnetic ion; in addition, the relative paramagnetic contribution to the spin–lattice relaxation time of

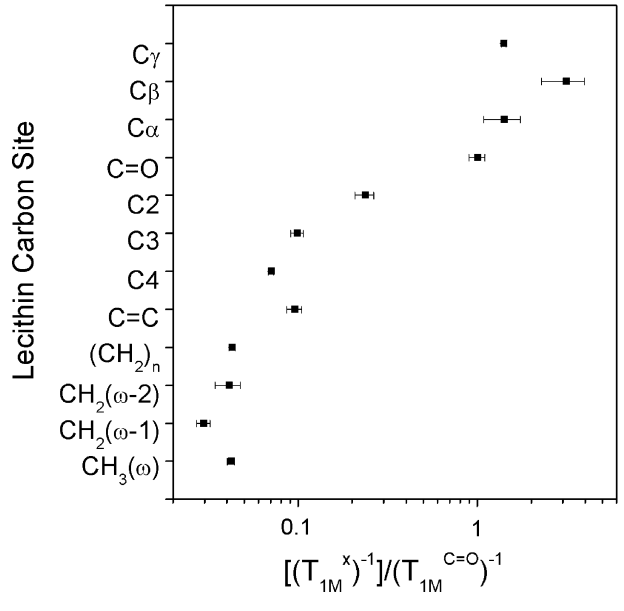

Fig. 3 Logarithmic plot of the paramagnetic contribution to ^{13}C spin–lattice relaxation times [normalized to $(T_{1M}{}^{C=O})^{-1}$ for EYL] of EYL in the presence of Triclosan (EYL/Triclosan molar ratio, 3:1)

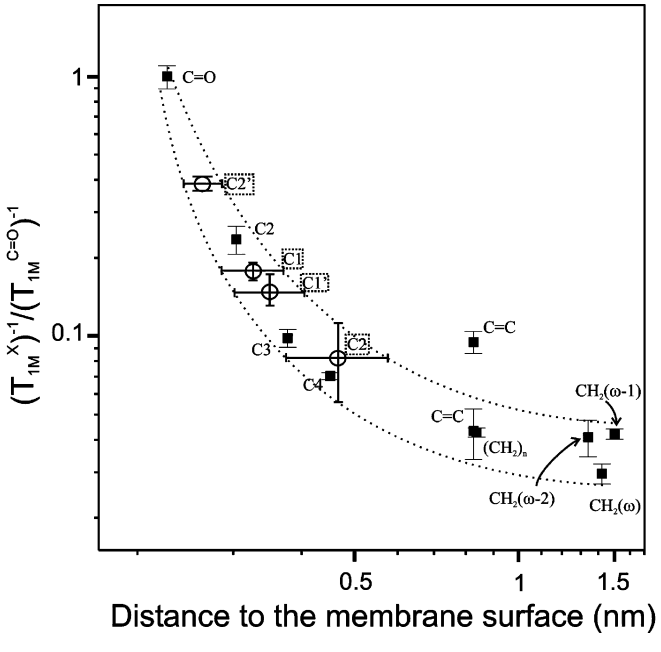

Fig. 4 Mean relative distance (nm) to the membrane surface for the carbon atoms of EYL (*solid squares*) and Triclosan (*open circles*) in MLVs containing EYL/Triclosan at a molar fraction of 3:1. See text for details

carbons $(CH_2)_n$ and CH = CH of the EYL molecule encompasses a relatively great region between C4 and $CH_2^{(\omega-2)}$ (Figs. 3 and 4). In order to calculate relative distances to the membrane surface, it was assumed that the hydrocarbon chains of the phospholipid molecule are considered as a single fragment, despite the fact that they are positionally non-equivalent in crystalline, gel, and liquid phases.

The experimental data actually represent the distance from the molecule of Triclosan to the paramagnetic ion rather than the distance to the membrane surface, which should be slightly different. The situation is further complicated by (1) the possibility that different Gd^{3+} ions might affect the same molecule so that the effect of Gd^{3+} ions on the carbon resonances of Triclosan would not depend strictly on their position along the EYL molecule, (2) the possibility that Triclosan does not reside at a fixed location in the MLV but instead oscillates between different positions in the membrane, (3) incomplete information on the mechanism by which paramagnetic ions (such as Gd^{3+}) affect spin–lattice relaxation times, and (4) distances from the different carbons to the phosphate may depend also on phospholipid composition and temperature (Villalaín 1996). Nevertheless, the method employed in this study provides very good estimates of the positions of Triclosan carbon atoms C1, C2, C1′, and C2′ relative to the carbon atoms of EYL in MLVs, and hence the overall orientation of Triclosan in relation to EYL. The results indicate an orientation of Triclosan vis-à-vis EYL, wherein Triclosan is confined near the polar headgroup region of EYL and aligned approximately parallel to the membrane surface (approximately orthogonal to the axis of the EYL hydrocarbon tail).

Bacterial growth inhibition by Triclosan does not depend upon loss of intracellular constituents but instead may result, at least in part, from subtle membrane structural perturbations that, while not drastic enough to perforate the membrane, nonetheless interfere with normal membrane functioning. We have shown previously (Villalaín et al. 2001) that Triclosan has a very high lipid/water partition coefficient and significantly affects the structure of phospholipid membranes in which it embeds; these include perturbation of membrane lipid packing, an increase in acyl chain motion accompanied by an increase in hydrophobic volume per molecule, changes in lipid polymorphism, and induction of immiscibilities (lateral phase separation) in both fluid and gel phases of the phospholipid membrane. The location and orientation of Triclosan in EYL MLVs inferred from the present work are consistent with the modulation of phospholipid membrane structure reported earlier and strongly suggests that, beyond any effect it may have on enzymes of the fatty acid biosynthetic pathway, the ability of Triclosan to weaken van der Waals interactions between adjacent phospholipid chains and disrupt membrane structures almost certainly contributes to the observed antibacterial action of this germicide.

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