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Interaction of antiaggregant molecule ajoene with membranes

An ESR and ^1H, ^2H, ^{31}P-NMR study

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Abstract. The structure of ajoene, a molecule extracted from garlic, has been studied by ^1H-NMR and its interaction with model membranes by ^1H-, ^2H-, ^{31}-P-NMR and ESR experiments. This study clearly shows that the ajoene molecule is located deep in the layer and is close to the interlayer medium. Moreover while NMR experiments show that the membrane structure is only slightly affected by the presence of ajoene, ESR experiments reveal significant modifications in phospholipid dynamics. This interaction, observed before with the phenothiazine derivative, promazine, results in an increase of the membrane fluidity in its hydrophobic part and could be related to clinical properties of ajoene.

Key words: Ajoene, drug-membrane interaction, NMR, ESR

Introduction

Ajoene (4-5-9 trithiododeca, 1-6-11 triene, 9 oxide), a molecule extracted from garlic, has been shown to confer protection against thrombosis and atherosclerosis by inhibiting platelet aggregation (Apitz-Castro et al. 1983; Appleton and Tansey 1975). Adetumbi and Lau (1983), Arunachalam (1980), Esanu (1981), Delaha and Garagusi (1985), Tansey and Appleton (1975), and Upadhyay (1980) showed that ajoene exhibits in vivo and in vitro antibacterial and antifungal properties. Membrane modifications (i.e. platelet membrane receptor exposure) or cell-wall alterations (Apitz-Castro et al. 1986; Block et al. 1984) of cells have been observed. Such properties, also observed in prokaryotic systems (Yoshida et al. 1987) suggest a general mechanism for interaction with a membrane. The present paper describes the study of the interaction of ajoene with model membranes. As a first step, the structure of ajoene in DMSO solution has been investigated by ^1H-NMR two dimensional experiments. ^1H-NMR experiments were also used to study the location of ajoene in the lipid bilayer of model membranes. The membrane structure in the presence of ajoene was investigated by ^{31}P and ^2H-NMR experiments. Finally ESR experiments were used to explore membrane dynamics in the presence of ajoene by using nitroxide labeled fatty acids.

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Material and methods

Ajoene and lipids

Synthetic ajoene was prepared as previously described (Apitz-Castro et al. 1986). L-α-phosphatidyl choline (PC) was extracted from egg yolk following the method of Patel and Sparrow (1979). Phosphatidic acid (PA), cholesterol (CH) and dimyristol-phosphatidylcholine (DMPC) were from Sigma. Spin labeled fatty acids (near the polar head -5NS-, or near the end of the acyl chain -16NS-) for ESR were from Molecular Probe.

Samples for NMR and ESR experiments

Small unilamellar vesicles (SUV): phospholipids (phosphatidylcholine and phosphatidic acid PC/PA 9 : 1) were lyophilized twice in D_2O and resuspended in pure D_2O of a final lipid concentration of 6 m*M*. SUV were formed by 1 h bath sonication. For ajoene containing SUV, lipids and ajoene (0.3 m*M*) were cosonicated after lyophilization.

Large unilamellar vesicles (LUV) for ESR experiments were prepared according to the method of Szoka and Papahadjopoulos (1978), filtered at 0.2 μm on polycarbonate porous membranes (Nuclepore Corps, Pleasanton, CA), for a final lipid concentration 20 m*M*. Lipid molar ratios were either PC/PA 9 : 1, or PC/PA/CH 8 : 1 : 1. Ajoene concentration was in the range 0 to 320 μ*M*.

DMPC liposomes for ^{31}P-NMR and ^{2}H-NMR experiments were prepared following Roux (1986). The suspension was degassed under nitrogen gas then introduced into NMR tubes and sealed. Liposomes were formed by five fast freezing and thawing cycles. Final lipid concentration was 50 m*M* and ajoene/lipid ratio was 1:30. For ^{2}H-NMR experiments lyophilized DMPC containing 10% perdeuterated phospholipids was resuspended in D_2O depleted water.

^{1}H-NMR experiments

Spectra were recorded at 500 MHz on a Bruker WM-500 spectrometer and referenced relative to internal tetramethylsilane (TMS). 2D-COSY, relayed coherence transfer 2D-COSY, and 2D-NOESY spectra were recorded using the standard pulse sequences: $(\pi/2-t1-\pi/2-t2)$, $(\pi/2-t1-\pi/2-T-\pi-T-\pi/2-t2)$ (Wagner 1983) and $(\pi/2-t1-\pi/2-Tm-\pi/2-t2)$ (Macura and Ernst 1980) with a mixing time *Tm* of 300 ms.

^{31}P and ^{2}H-NMR experiments

^{31}P and ^{2}H NMR experiments were performed at 121.5 and 46 MHz respectively on a Bruker MSL-300 spectrometer. Phosphorus spectra were recorded at 34°C by using a dipolar echo sequence $(\pi/2-t-\pi-t)$ with a t value of 20 µs, a dwell time of 10 µs and a two-level broadband proton decoupling. Phosphoric acid was used as external reference. Deuterium spectra were recorded at 34°C by using a quadrupolar echo sequence $(\pi/2-t-\pi/2-t)$ with a t value of 100 µs and a dwell time of 2 µs. The free induction decay was shifted by fractions of the dwell time to ensure that its effective time for the Fourier transform corresponds to the top of quadrupolar echo (Davis et al. 1976, 1983). Oriented ^{2}H-NMR spectra were obtained by the numerical de-Pakeing procedure described by Sternin (1982).

ESR experiments

For ESR experiments, spin-labeled fatty acids were incorporated into large unilamellar vesicles. The spectra were recorded on a Varian E109 spectrometer in a temperature range from 0° to 50°C. For 16NS experiments the correlation time τ_c, directly related to the membrane fluidity, was calculated by the following relation:

$$\tau_c = 6.65\,10^{10}\,\delta H_0\,((H + 1/H_0)^{1/2}-1),$$

where δH_0 represents the central signal width, H_0 its intensity and $H+1$ the lowfield signal intensity. The slope of the curve obtained by plotting Log V^+ (the reciprocal correlation time, $1/\tau_c$) versus reciprocal absolute temperature gives the activation energy of the apparent membrane viscosity. Since the previous relation cannot be used for long correlation times, spectra measured with 5 NS fatty acids were analysed by measuring the $2T'_{\parallel}$ parameter, which is the spectral distance between lowfield and highfield resonances.

Results and discussion

Ajoene in DMSO and in model membranes

Ajoene structure in DMSO. Figure 1 shows the 500 MHz ^{1}H-NMR spectrum of ajoene (50 m*M* in DMSO-D 6 at 42°C). Proton assignment was achieved as follows. As expected two isomers (*cis, trans*) are present since two doublets can be assigned to the H 5 proton (proton labeling is given in Fig. 1). H 5 *cis-Z* form-resonance is characterized by a J (H 5–H 4) coupling constant of 9.8 Hz located at 6.56 ppm while the H 5 *trans-E* form-14 Hz doublet is found at 6.38 ppm, in agreement with the previous assignment (Block 1985). Since the CH_2 (2), (3), and (6) protons as well as the two methylene terminal groups exhibit close chemical shifts (about 3.4 ppm and 5.3 ppm respectively) we used a RCT-COSY experiment to distinguish all the resonances (Fig. 1). Dipolar correlations observed on the NOESY spectrum (not shown) are consistent with a folded conformation of the molecule since close contacts exist between protons of the two terminal groups (0,8) and also between H 4 and H 7 protons in the case of the *Z* isomer. Well resolved spectra recorded for one dimensional NOE experiments confirm that both isomers adopt a folded conformation.

In order to study the location of ajoene in membranes we used sonicated unilamellar vesicles (SUV) as a membrane model. Such a system gives relatively narrow lines and by using a high field spectrometer (500 MHz) one can expect to detect the drug resonances.

Ajoene location in membranes (SUV). Figure 2 A shows the 500 MHz ^{1}H-NMR spectrum of SUV in the presence of ajoene (lipid to drug ratio $R = 20$). An expanded difference spectrum obtained from spectrum 2 A and a pure SUV spectrum reveals that the phospholipid signals are not affected by the presence of ajoene and enables us to observe the ajoene resonances (Fig. 2 B) except for the highfield signals (3.36 ppm) whose detection is perturbed by the intense choline peak. The chemical shift values of all the drug signals are close to those found in DMSO. As far as the linewidths are concerned, the various ajoene resonances in SUV are broadened by comparison with the

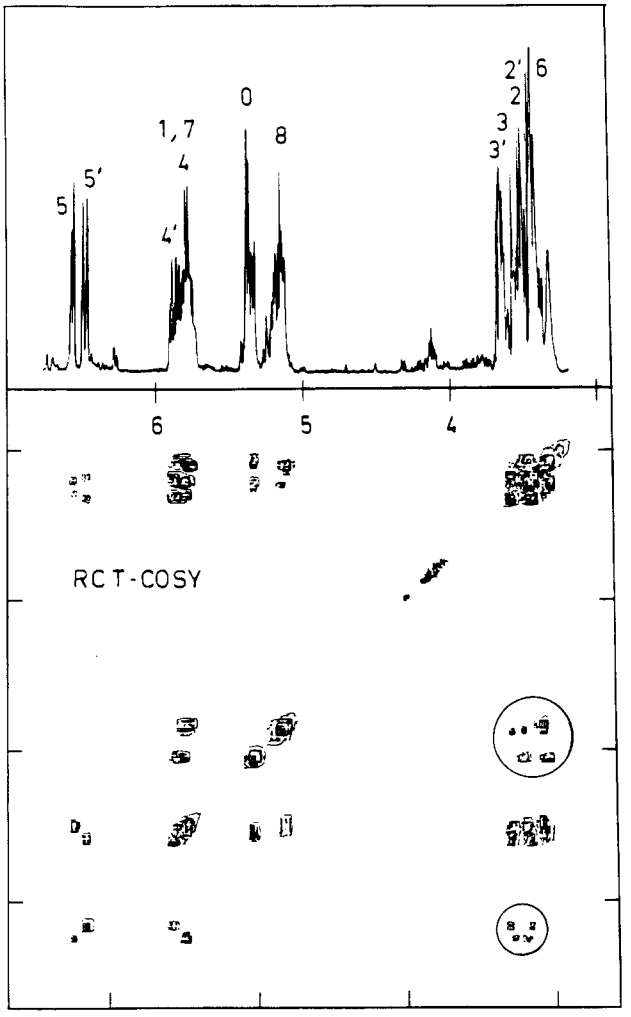
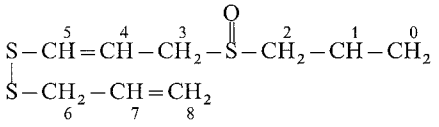


Fig. 1. 500 MHz ^{1}H-^{1}H RCT COSY matrix of ajoene (50 m*M*, DMSO, 42 °C) and its projection. Proton resonances are labeled as follows:



H 5 protons of the *Z* and *E* forms are labeled 5 and 5′ respectively. Cross peaks located inside the two circles are not observed in a COSY spectrum and correpond to second order connectivties. i.e. H (5)–CH_2 (3)

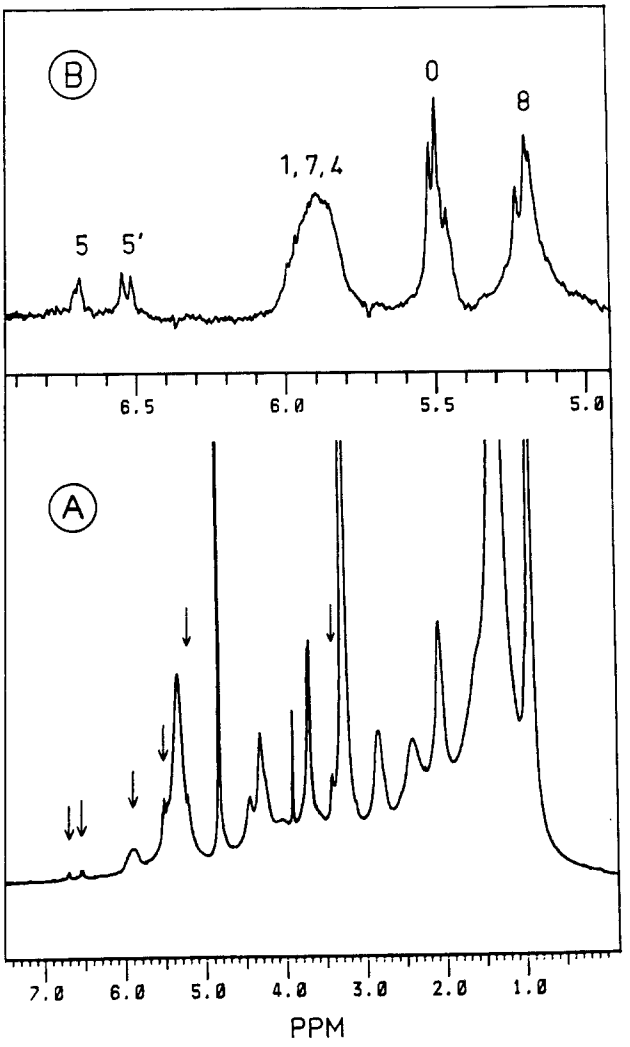


Fig. 2 A and B. 500 MHz ^{1}H-NMR: **A** spectrum of ajoene containing SUV. *Arrows* indicate ajoene resonances. **B** Expanded difference spectrum obtained by subtraction of spectrum 2 A with a pure SUV spectrum

spectrum in DMSO. The largest broadening effect is observed on the peak containing the H 1, H 4 and H 7 resonances and can be estimated to be 20 Hz. The H 5 (*E* and *Z*), H 0 and H 8 resonances exhibit a broadening value of about 10 Hz. By comparison with the phospholipid resonances, the linewidths of ajoene resonances are close to those observed for the protons of the most mobile part of the phospholipid molecule, i.e. the extremities. In order to precisely locate the ajoene molecule in the bilayer, we added progressive amounts of paramagnetic manganese ions to ajoene containing SUV.

First it is important to recall some basic results concerning the effect induced by Mn ions on SUV phospholipid resonances (Chan et al. 1971). In the case of the polar headgroup, for which each proton gives rise to a separate resonance for each layer, the addition of a small amount of Mn ions is sufficient to broaden beyond detection the signal corresponding to the external layer while that corresponding to the internal layer is unaffected. In contrast, the terminal methyl protons appear as a single resonance which is progressively broadened on increasing the Mn concentration (Fig. 3). In order to follow the broadening of the ajoene resonance upon addition of Mn ions, we performed two sets of experiments on SUV in the absence and in the presence of ajoene and then observed the drug resonances by spectrum difference. In fact only the lowfield H 5 resonance can be clearly analysed. The H 5 linewidth variation versus Mn concentration is

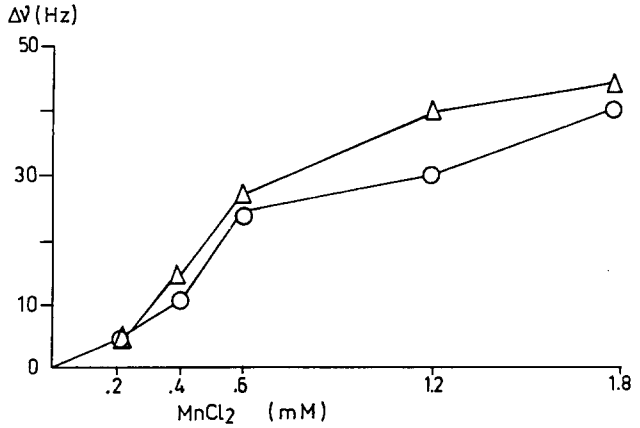


Fig. 3. 500 MHz ^{1}H-NMR of SUV: resonance broadening (Hz) of Phosphatidylcholine terminal methyl protons (o), and of H 5 proton of ajoene (Δ) plotted versus $MnCl_2$ concentration

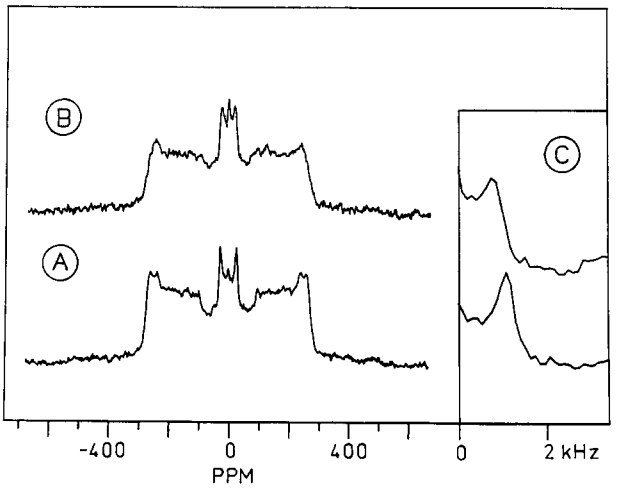


Fig. 4. 46 MHz ^{2}H-NMR spectra (quadrupolar echo) of DMPC liposomes in the absence (**A**) and in the presence (**B**) of ajoene at 34°C. *Insert* (**C**) shows the expanded region containing the terminal methyl signal of both spectra

plotted in Fig. 3 and is practically superimposed on the curve corresponding to the phospholipid terminal methyl protons.

This study clearly shows that the ajoene molecule is located deep in the layer, and the linewidth values suggest that the drug is close to the interlayer medium. In the next section, we investigate the influence of the ajoene molecule on the bilayer structure by using liposomes as a membrane model (as mentioned above, SUV resonances are unaffected by the presence of ajoene) and ^{31}P- and ^{2}H-NMR spectroscopies. Such techniques are well known to be powerful for studying drug-lipid interactions (Zidovedzki et al. 1988).

Influence of ajoene on membrane structure and dynamics

^{31}P- and ^{2}H-NMR experiments. Pure DMPC exhibits a typical axially symmetric powder phosphorus spectrum and a chemical shift anisotropy of 43 ppm in agreement with a standard lipid bilayer structure (Gorenstein 1984). The spectrum recorded in the presence of ajoene shows a quite similar spectrum and the chemical shift anisotropy is not modified. Therefore one can conclude that the main bilayer structure is conserved in the presence of ajoene.

In accordance with the phosphorus spectra, ^{2}H-NMR spectra of perdeuterated DMPC in the presence or in the absence of ajoene (Fig. 4 A and B) appear as a superimposition of Pake doublets corresponding to the various CD_2 groups of the lipid acyl chain (Seelig 1977). The outer quadrupolar splitting corresponds to the methylenic deuterons close to the glycerol group and the inner one to the terminal methyl group. The only significant effect of ajoene is the decrease of the quadrupolar splitting relative to the terminal methyl group (Fig. 4 C) which is reduced from 2,400 to 1,800 Hz (measured after de-Pake-ing). Such a reduction of the quadrupolar splitting of the methyl resonance could be related to our previous ^{1}H-NMR study which enables us to localize the drug in the interlayer medium.

The ^{31}P-NMR and ^{2}H-NMR study shows that the average membrane structure is only slightly affected by the presence of ajoene. In order to investigate phospholipid dynamics in the presence of the drug we performed ESR experiments using spin labeled fatty acids.

Influence of ajoene on phospholipid dynamics. Dynamic properties of membranes in the presence of ajoene were observed using cholesterol-containing, or sterol free LUV. Nitroxide-labeled fatty acids are spontaneously incorporated into membranes: the 16 NS-ESR study investigates the depth of the lipidic bilayer and the 5 NS-ESR experiments give information on the hydrophilic part of the membrane. In sterol free vesicles, ESR experiments performed with 5 NS detect no significant difference in the $2T' \parallel = f(1/T°C)$ curve (see methods) when ajoene is added. In contrast 16 NS-ESR experiments clearly show that the presence of ajoene increases the fluidity of hydrocarbon chains (Fig. 5); addition of 150 μ*M* ajoene leads to an increase of the Log $V^+ = f(1/T)$ curve slope in the whole temperature range. Moreover the curve is significantly shifted to the high frequency domain.

A second set of 16 NS ESR experiments was performed with cholesterol-phospholipid LUV. The well-known condensing influence of cholesterol on membrane dynamics (in the experimental temperature range) is clearly observed (Fig. 5) (Cullis et al. 1975). Nevertheless, addition of ajoene induces similar effects

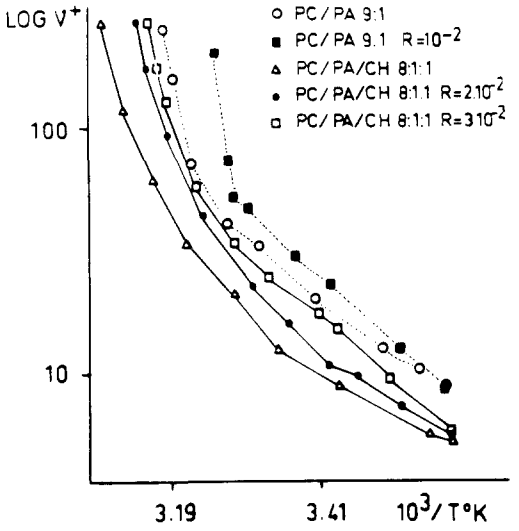


Fig. 5. Semi-log plot of V^+ (reciprocal correlation time) versus reciprocal absolute temperature obtained from ESR measurements of 16 NS fatty acid incorporated into LUV (with and without cholesterol) in the presence and in the absence of ajoene (see text). R is ajoene/lipids molar ratio

to those revealed in sterol free vesicles, in particular the significant shift of the Log $V^+ = f(1/T)$ curve, but larger amounts of ajoene (320 μM) are required.

Therefore, ajoene appears as a liquefying reagent whose interactions with the phospholipid molecules are localized deep in the layer, since 5 NS-ESR experiments detect no perturbation in close agreement with our NMR results.

Concluding remarks

The present study shows that the ajoene molecule in membranes is close to the interlayer medium and induces a disordering effect on the phospholipid packing without any significant perturbation of the main bilayer structure. Many small molecules such as local anesthetics or analgesics incorporated into membranes exhibit various modes of interaction. Ajoene which interacts with the bilayer structure without a direct permeabilizing effect can be compared to these molecules.

Amphiphilic analgesics – e.g. tetracaïne – incorporated into membranes modifiy the organization of both hydrophilic and hydrophobic parts of the bilayer (Siminovitch et al. 1984). Conversely, Thewalt et al. (1985) showed that the alkanol-class of analgesics do not significantly affect the main bilayer structure. Furthermore, general hydrophilic anesthetics – e.g. halothane – exclusively interact with the polar head group region of the membrane (Shieh et al. 1976). Ajoene increases the fluidity of the hydrocarbon chains but unlike tetracaïn, does not modify the hydrophilic part of the bilayer. This property was found before with a phenothiazine derivative, promazine (Leterrier et al. 1976; Seeman 1972).

ESR experiments performed on erythrocytes and platelet membranes show a desordering effect induced by ajoene in the deep part of the membrane (unpublished) as in the case of modal membranes. Thus this mode of interaction seems independent on the nature of the membrane.

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