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Topography of alphaxalone and Δ^{16}-alphaxalone in membrane bilayers containing cholesterol

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Abstract

We have used small-angle X-ray diffraction and differential scanning calorimetry (DSC) to study the topographies of alphaxalone and its biologically inactive analog Δ^{16}-alphaxalone in dimyristoylphosphatidylcholine (DMPC) and DMPC/cholesterol model membranes. Diffraction patterns were obtained and analyzed for preparations of bilayers without and with the steroids. Temperature dependence of the total period repeat distance (*d*-spacing) allowed us to identify equivalent temperatures at which the preparations had similar *d*-spacing and were in the same mesomorphic state. The combination of X-ray and DSC data showed that the anesthetic steroid alphaxalone broadens the membrane phase transition and increases the ratio of *gauche:trans* conformers in the membranes in contrast to the inactive steroid Δ^{16}-alphaxalone which affects the membranes only marginally. In model DMPC membranes alphaxalone and Δ^{16}-alphaxalone are located near the bilayer interface. This location is maintained by alphaxalone when cholesterol is incorporated in the bilayer as evidenced by the X-ray measurements. However, when Δ^{16}-alphaxalone is incorporated in cholesterol containing bilayers, a decrease in the electron density profile of the preparation is observed. This can be explained by invoking the formation of a Δ^{16}-alphaxalone -cholesterol complex. The Δ^{16}-alphaxalone complex shows no periodicity and is therefore, not detected in the X-ray diffraction experiment. Presumably, this complex forms aggregates either on the surface or inside the bilayer. This explanation corroborates DSC results which show that Δ^{16}-alphaxalone sharpens the phase transition of DMPC/cholesterol preparations, an indication that some cholesterol is excluded from the bilayer preparation after the addition of the biologically inactive steroid.

Key words: Small-angle X-ray diffraction; X-ray diffraction, small-angle; DSC; Drug-membrane interaction; Steroid; Alphaxalone; Δ^{16}-Alphaxalone

1. Introduction

Structure activity relationships (SAR) showed that anesthetic activity by steroids is subject to strict stereochemical requirements. Alphaxalone (5α-pregnan-3α-ol-11,20-dione) is a potent anesthetic steroid and was used clinically as the main active component in the commercially available anesthetic Althesin. However, Δ^{16}-alphaxalone (5α-pregn-16-en-3α-ol-11,20-dione), which differs from alphaxalone only by having a double bond in the C-16 position (see structures in Fig. 1), lacks anesthetic activity [1,2].

To account for this structural specificity, two theories have been developed. The first suggests that anesthetic steroids act after binding to a distinct site on a target membrane protein [3] or on the $GABA_A$ receptor complex [4–6]. The second hypothesizes that the sites of action are membrane lipids capable of a high degree of structural discrimination. Supporting evidence for this hypothesis has been obtained from electron spin resonance (ESR) experiments with spin-labeled bilayers containing cholesterol [7] and from our studies using high resolution ^{1}H-, ^{2}H-, ^{13}C-and solid state ^{2}H- and ^{13}C-NMR [8–10]. The ESR experiments showed a correlation between the anesthetic potency with the steroid's ability to disorder spin-labeled liposomes. Thus, steroids with anesthetic activity fluidized the model membrane to a higher degree compared to their inactive analogs. High resolution ^{1}H-, ^{13}C- and

Abbreviations: DSC, differential scanning calorimetry; DMPC, dimyristoylphosphatidylcholine; RH, relative humidity.

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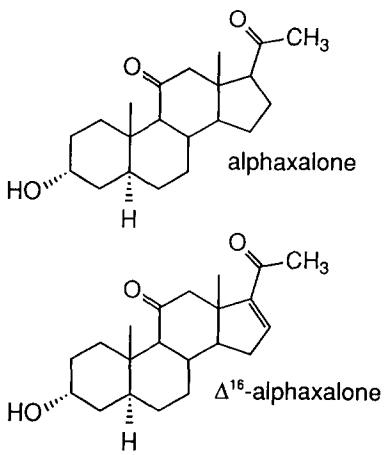

Fig. 1. Chemical structures of alphaxalone and Δ^{16}-alphaxalone.

^{2}H-NMR spectroscopy results showed that alphaxalone is considerably more mobile in the liquid crystalline state of the bilayers compared to its inactive congener Δ^{16}-alphaxalone [8]. Solid state ^{2}H-NMR in multilamellar bilayer dispersions of dimyristoylphosphatidylcholine with perdeuterated acyl chains (DMPC-d_{54}) showed that alphaxalone decreased the quadrupolar splittings in the spectrum while Δ^{16}-alphaxalone produced no change. This observation was interpreted to mean that alphaxalone increases the *gauche : trans* conformer ratio of the acyl chains of DMPC whereas Δ^{16}-alphaxalone does not perturb the lipid chains. The above preferential effects of alphaxalone in the bilayer were attributed to the differences in molecular geometry between the two molecules. Indeed, conformational analysis using high resolution NMR data showed that the anesthetic steroid alphaxalone has its D-ring puckered and the 17-acetyl group protruding from the β-face of the steroid. In contrast, the inactive steroid has the D-ring almost flat with the acetyl side-chain in the S-*trans* conformation [8]. Similar results were obtained with aqueous multilamellar dispersions of DPPC with specific ^{13}C and ^{2}H labels which acted as endogenous probes [9,10].

As a sequel of the previous work, this study seeks to determine the topography of alphaxalone and Δ^{16}-alphaxalone in model DMPC and DMPC/cholesterol membranes using small-angle X-ray diffraction as well as the different effects of the two steroids on the thermotropic properties of the corresponding bilayers.

2. Materials and methods

2.1. Materials

Alphaxalone and Δ^{16}-alphaxalone were kindly donated by Glaxo Group Research, Middlesex, UK. DMPC and cholesterol were obtained from Avanti Polar Lipids, Birmingham, AL, USA.

2.2. Sample preparation

Identically prepared partially hydrated membrane preparations were used for both the X-ray diffraction and DSC experiments. DMPC (1 mg for X-ray diffraction or 7 mg for DSC) alone or with the appropriate amount of alphaxalone or Δ^{16}-alphaxalone was dissolved in chloroform (99 + % pure, Aldrich, Milwaukee, WI, USA). The solvent was then removed using a stream of O_2-free N_2 and the samples were dried under vacuum (0.1 mmHg) for 6 h. After adding distilled water, each sample was vortexed and sonicated in a water bath (50°C) for 15 min until the suspension was translucent. For the X-ray diffraction experiments, the preparations were deposited on an aluminum foil, dried at 35°C and mounted on a curved glass holder. For the DSC experiments, the vesicles were deposited on a glass surface, dried at 35°C and scraped into a stainless steel DSC capsule (0.25 cm height × 0.5 cm diameter). Prior to the X-ray and DSC experiments, samples were suspended in closed vials (5 cm height × 3 cm diameter) with regulated relative humidity (RH) 98% using saturated K_2SO_4 solution for 24 h and 48 h, respectively. This produces *partially hydrated* multilamellar vesicles. For the X-ray experiments, the preparations were transferred into RH-regulated canisters (5 cm height × 3 cm diameter) 3–5 h before the experiments. To keep the partial hydration of the DSC samples constant, the samples were sealed hermetically in the stainless steel capsules. Partially hydrated samples prepared in this manner gave identical thermograms when tested repeatedly over a seven-day period, indicating that there was no change in the water content of the sample.

2.3. Differential scanning calorimetry

Thermograms were obtained on a Perkin-Elmer DSC-7 instrument. Prior to scanning, samples were held above their phase transition temperature for 5 min to ensure complete equilibration. All samples were scanned at least twice until identical thermograms were obtained. Heating and cooling scans gave similar results. Only beyond 5.0 K/min did the scanning rate affect the thermograms by slightly broadening the breadth of the phase transition and shifting the main phase transition. Thus, a scanning rate of 1.5 K/min was used. The temperature scale of the calorimeter was calibrated using a fully hydrated dipalmitoylphosphatidylcholine (DPPC) preparation and pure crystalline indium as standard samples. The effect of the equilibration time was also examined. Identical ther-

mograms were obtained if more than 48 h equilibration time was allowed.

2.4. X-ray diffraction

X-ray diffraction experiments were carried out with an Elliott GX18 generator (Marconi Avionics), equipped with a camera utilizing a single vertical Franks' mirror [11]. Small-angle X-ray diffraction patterns were collected using a Braun position sensitive proportional counting gas flow detector (Innovative Technology, South Hamilton, MA, USA). Sample temperature was kept constant with a Neslab 4B circulating water bath (Neslab Instruments, Newington, NH, USA), and monitored with a thermister probe placed next to the canister. During the experiment a helium path was used to prevent background scatter for a specimen-to-detector distance of 130 mm and the diffraction data was collected with digital accumulations of $1 \cdot 10^6$ to $2 \cdot 10^6$ counts to improve the signal-to-noise ratio. Finally, data was transferred to a VAX computer system and the intensities were integrated directly from the computer plots by calculating the area under the diffraction peaks.

3. Results and discussion

3.1. Differential scanning calorimetry

Representative thermograms from DMPC preparations equilibrated at RH 98% and from similar preparations containing alphaxalone and Δ^{16}-alphaxalone are shown in Fig. 2. Thermogram A is from the DMPC preparation without drug and thermogram B shows the effect of alphaxalone in DMPC bilayers (lipid-to-steroid molar ratio 0.94 : 0.06 or $x = 0.06$). The presence of the biologically active drug lowers the phase transition temperature by 5 K and broadens this transition. Conversely, the inactive Δ^{16}-alphaxalone (C) only lowers the phase transition by 2 K and does not broaden it. At a higher concentration ($x = 0.10$), alphaxalone lowers the phase transition temperature even more and increases the breadth of the phase transition. Conversely, at $x = 0.10$, Δ^{16}-alphaxalone does not further affect the phase transition peak temperature and induces only a small broadening of this transition. Overall, the biologically active steroid induces more pronounced effects on the thermotropic properties of the model membrane than the inactive analog. These results are in agreement with the previously presented data using fully hydrated phosphatidylcholine model membranes [12].

Thermograms from DMPC/cholesterol ($x = 0.15$) bilayers containing each of the two analogs were also obtained (see Fig. 3). The phase transition as observed

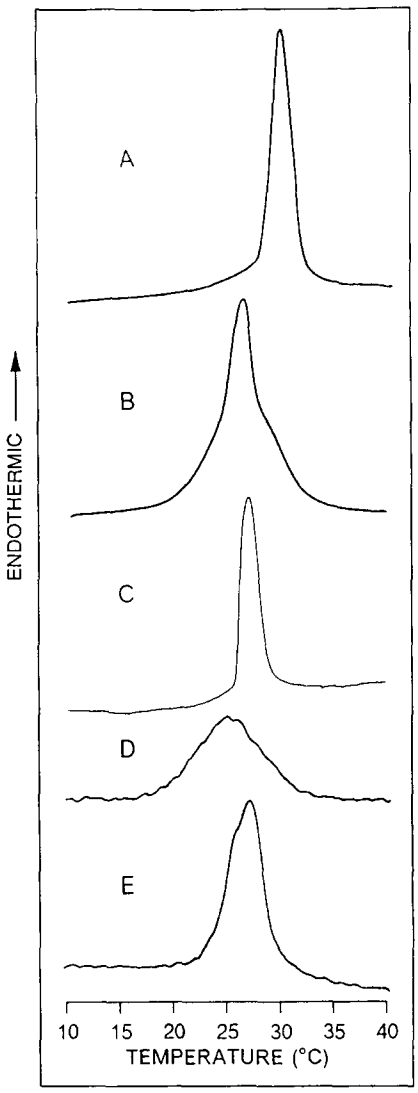

Fig. 2. Differential scanning calorimetry thermograms of partially hydrated bilayers equilibrated at RH 98%. (A) DMPC alone; (B) DMPC + alphaxalone ($x = 0.06$); (C) DMPC + Δ^{16}-alphaxalone ($x = 0.06$); (D) DMPC + alphaxalone ($x = 0.10$); (E) DMPC + Δ^{16}-alphaxalone ($x = 0.10$).

in thermogram A is abolished by the addition of the active anesthetic steroid as shown in thermogram B. Conversely, addition of the inactive analog has an opposite effect on the same preparation. Instead of broadening the phase transition, the drug dramatically sharpens it and, as a result, the main peak in thermogram C is now sharper than the one in A. Presumably, this happens because Δ^{16}-alphaxalone excludes cholesterol from DMPC bilayers, creating a new unknown phase of Δ^{16}-alphaxalone/cholesterol complex. This sharp endotherm represents a phase transition of DMPC bilayers containing a reduced amount of cholesterol less than $x = 0.15$ and may be some small quantity of Δ^{16}-alphaxalone.

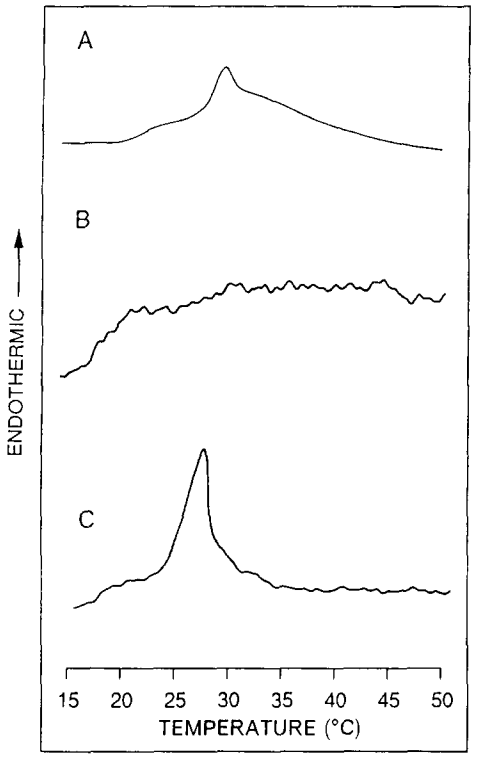

Fig. 3. Thermograms of partially hydrated bilayers at RH 98% (A) DMPC + cholesterol ($x = 0.15$); (B) DMPC + cholesterol ($x = 0.15$) + alphaxalone ($x = 0.10$); (C) DMPC + cholesterol ($x = 0.15$) + Δ^{16}-alphaxalone ($x = 0.10$).

3.2. Small-angle X-ray diffraction

The small-angle X-ray experiments involved using DMPC preparations containing alphaxalone and Δ^{16}-alphaxalone. The analysis of the small-angle X-ray diffraction results involved extraction of the total period repeat distance (d-spacing) using Bragg's Law, determination of the signs of the structure factors using the bilayer swelling approach [13,14] and calculation of the electron density profile using a Fourier transform algorithm. At the end, an approach was used in which a step-function equivalent profile was calculated for DMPC bilayers and placed on the absolute electron density scale [15–17]. Information on the location of the molecules in the bilayer was obtained by comparing the electron density profiles of the DMPC or DMPC/cholesterol preparations without and with the steroids.

The preparations were compared in the liquid crystalline phase where they had the same number of diffraction orders ($h = 4$) and at equivalent temperatures where they had identical d-spacings.

3.3. Temperature dependence of d-spacing

In order to determine the equivalent temperatures at which comparison of the electron density profiles of the three preparations could be made, d-spacings changes at the transition between the two mesomorphic states of the lipid bilayers were monitored. Comparisons were then made by using an approach in which a step-function equivalent profile was calculated for DMPC bilayers and placed on the absolute electron density scale [15–17].

As is characteristic for such systems the d-spacing decreases when the bilayer undergoes the gel to liquid-crystalline phase transition and continues to decrease gradually as the temperature rises above the transition. This is due to conformational changes in the bilayer at the phase transition temperature, which result in an increase of the *gauche : trans* conformer ratio in the lipid chains.

At all temperatures in both DMPC (Fig. 4A) and DMPC/cholesterol mixtures (Fig. 4B), the d-spacings of bilayers containing the biologically active steroid, consistently were found to be 1.5 Å lower than those of the Δ^{16}-alphaxalone-containing bilayers. The d-spacing curves representing the two steroid-containing preparations are essentially parallel to each other. Also, at temperatures above the phase transition, the preparation containing Δ^{16}-alphaxalone has exactly the same d-spacings as the drug-free control preparation.

Comparison of the d-spacing of the preparations of DMPC and DMPC/cholesterol above the phase transition showed that the presence of cholesterol increases the d-spacing. This confirms that cholesterol decreases the *gauche : trans* conformer ratio in the lipid chain when the bilayer is in the liquid-crystalline phase.

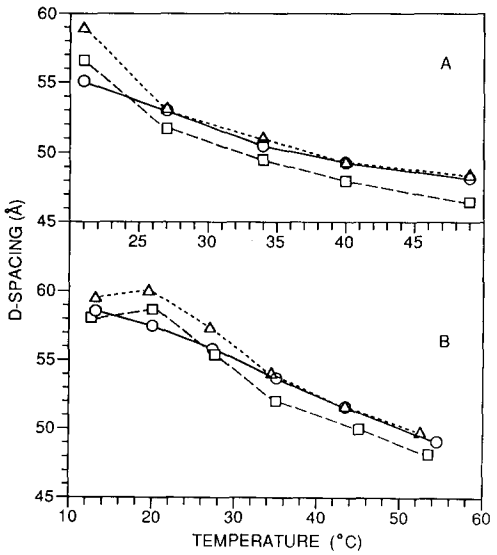

Fig. 4. d-Spacing as a function of temperature for bilayers of DMPC (circles and solid lines), DMPC + alphaxalone ($x = 0.10$, squares and dashed lines), and DMPC + Δ^{16}-alphaxalone ($x = 0.10$, triangles and dotted lines) at RH 98% (A) without cholesterol; (B) with cholesterol ($x = 0.15$).

3.4. The topography of alphaxalone in membrane bilayers

Properly superimposed electron density profiles of DMPC and DMPC + alphaxalone (with and without cholesterol) are shown in Fig. 5 where z is the distance across the bilayer, perpendicular to its plane, measured from the bilayer center. The electron density profile correlates the molecular features of the bilayer with its corresponding electron density values. In each electron density profile, the middle trough corresponds to the terminal methyl group of the acyl chains while the maxima which occur at about 18 Å from the center correspond to the phosphates of the head groups. The electron density profiles of DMPC and DMPC + alphaxalone preparations were superimposed assuming identical electron densities at the highest and lowest points of the profiles. These two points were chosen for normalization because earlier experimental data showed the anesthetic steroids to be located at the level of the bilayer acyl chains and do not exert significant effects on the head group.

The comparison revealed an electron density difference near the bilayer interface in the region centered at $z = 11.2$ Å. This can clearly be seen in the magnified vertical difference scale below the superposition. This increase should be due to the presence of alphaxalone and, therefore, the peak position corresponds to the location of the center of mass of alphaxalone in the DMPC bilayer. The width of the difference peak is of the order of 14 Å, which is approximately the length of the long axis of the alphaxalone molecule, indicating that, in all likelihood, the molecule orients with its long axis parallel to the lipid chains. The location of alphaxalone in the bilayer as revealed by the X-ray data positions the molecule in a manner which allows its 3α-hydroxy group to be in the proximity of the carbonyl groups of the DMPC acyl chains.

The presence of cholesterol in the DMPC preparations produces changes in the electron density profile. However, if superposition of two DMPC/cholesterol preparations with and without alphaxalone is carried out using criteria similar to those described above, analogous results are obtained. The cholesterol-containing preparations give a difference plot from the electron density profiles with a peak centered at $z =$ 13.2 Å, approximately 2 Å closer to the interface when compared to the preparation without cholesterol. Also the width of the difference peak is now 16 angstroms, i.e., larger by 2 Å. The data from the second set of preparations indicate that cholesterol causes a slight shift in the time-averaged location of the steroid closer to the interface although still maintaining its proximity with the carbonyl groups.

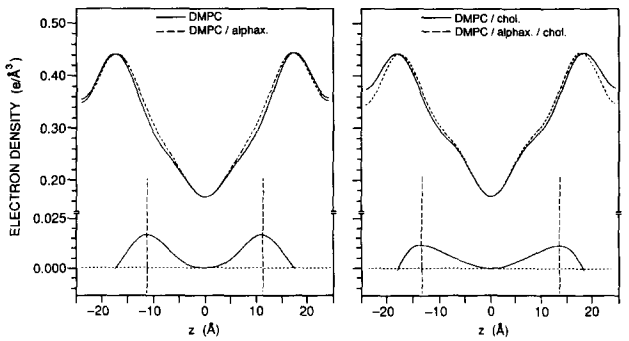

Fig. 5. Left: Comparison of electron density profiles between DMPC (solid line) at 49°C and DMPC + alphaxalone ($x = 0.10$) at 40°C and RH 98%. Right: Comparison of electron density profiles between DMPC + cholesterol ($x = 0.15$, solid line) at 54°C and DMPC + alphaxalone ($x = 0.10$) + cholesterol ($x = 0.15$) at 54°C. Below each superposition is the difference between the two profiles.

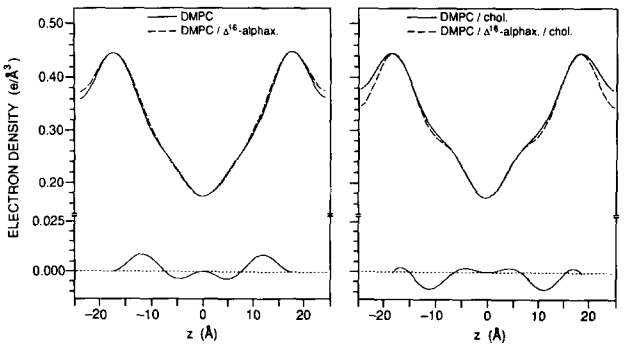

Fig. 6. Left: Comparison of electron density profiles between DMPC (solid line) at 49°C and DMPC + Δ^{16}-alphaxalone ($x = 0.10$) at 40°C and RH 98%. Right: Comparison of electron density profiles between DMPC + cholesterol ($x = 0.15$, solid line) at 54°C and DMPC + Δ^{16}-alphaxalone ($x = 0.10$) + cholesterol ($x = 0.15$) at 54°C. Below each superposition is the difference between the two profiles.

3.5. Topography of Δ^{16}-alphaxalone in the membrane bilayers

The electron density profiles of the DMPC and DMPC + Δ^{16}-alphaxalone preparations were superimposed also by assuming identical electron densities at the highest and lowest points of the profiles. The comparison revealed (Fig. 6) an electron density difference near the bilayer interface and in the same region as alphaxalone. However, the enhancement of the electron density profile was less pronounced, probably due to the lesser incorporation of the inactive anesthetic in the bilayer. Recent solid state ^{2}H-NMR data corroborate this speculation [12]. Superposition of two DMPC/cholesterol preparations with and without Δ^{16}-alphaxalone showed no electron density enhancement due to the inactive anesthetic but instead some decrease in the electron density of the steroid analog was observed (Fig. 6). In conjunction with the DSC results, the above data can be explained by invoking a Δ^{16}-alphaxalone complex with cholesterol. Such a Δ^{16}-alphaxalone complex shows no periodicity and is there-

fore, not detected in the X-ray diffraction experiment. Presumably, this complex forms aggregates either on the surface or inside the bilayer.

4. Conclusions

DSC studies show that alphaxalone perturbs model membranes while its inactive analog does only marginally. The decrease of *d*-spacing with increasing temperature shows that these perturbations also involve an increase in the ratio of *gauche : trans* conformers in the bilayer chains. These results are in agreement with the previous reported high resolution and solid state NMR data.

The ability of the anesthetic steroid to induce perturbations is related to its stereochemical characteristics which are responsible for an imperfect steroid-bilayer packing [7–10]. According to this model, the anesthetic steroid undergoes amphipathic interactions with the membrane bilayers and anchors itself at the interface with its 3α-hydroxy group near the carbonyl groups with which it may form a hydrogen bond. These amphipathic interactions are accompanied by a reduced lipid chain cooperativity.

While Δ^{16}-alphaxalone appears to anchor also at the interface of the membrane bilayers, it does not significantly perturb these bilayers. The data obtained here suggest that one of the reasons for its biological inactivity is the 'snug' packing of the anesthetic analog within the lipids which results in no significant membrane perturbation, a model we have discussed in earlier work [9,12].

In DMPC/cholesterol membranes which are more biologically relevant, Δ^{16}-alphaxalone appears to complex with the membrane cholesterol. Such an aggregate of the steroid with cholesterol can be located either at the surface or in the interior of membrane bilayer. The aggregate shows no periodicity and is not observed in the X-ray diffraction experiment.

The emerging picture is that the anesthetic steroid alphaxalone perturbs the membrane lipids and thus by affecting the functions of some important membrane-associated proteins, eventually leads to anesthesia. However, Δ^{16}-alphaxalone fails to significantly perturb the lipid bilayer, an observation which may explain its biological inactivity.

Acknowledgments

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