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EFFECTS OF CYCLOARTENOL AND LANOSTEROL ON ARTIFICIAL AND NATURAL MEMBRANES

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SUMMARY: Cycloartenol, a 9,19-cyclopropane sterol which is isomeric with lanosterol, showed an ability intermediate between lanosterol and cholesterol to increase the microviscosity of lecithin vesicles, to serve as a growth factor for the sterol auxotroph Mycoplasma capricolum, and to increase the microviscosity of M. capricolum membranes. The corresponding membrane effects of cyclolaudenol which contains a methyl group added to C-24 of the isooctenyl side chain of cycloartenol are more like those shown by lanosterol. We propose that the enhanced effectiveness of cycloartenol over lanosterol is due to a more favorable spatial disposition of the angular 14α-methyl group on the α-face of the molecule promoting more effective van der Waals contacts between the phospholipid fatty acyl chains and the sterol α-face. Side chain alkylation appears to perturb such contacts, reducing the effectiveness of cyclolaudenol for competent membrane function in M. capricolum.

According to current views, cholesterol entering membrane bilayers aligns in parallel with the extended acyl chains of the phospholipid, the sterol hydroxyl group pointing toward the water-bilayer interface and the isooctyl side chain toward the bilayer interior (1). Also, there appears to be consensus that hydrophobic interactions between the sterol nucleus and the proximal segment (C-2 to C-10) of the phospholipid acyl chains are primarily responsible for modulating the physical state (fluidity or viscosity) of biomembranes (2).

Recent studies in this laboratory have emphasized the importance of sterol α-face planarity for phospholipid interactions. In particular, it has been stressed that this planarity is achieved in biological systems by

ABBREVIATIONS: Ptd Cho - phosphatidylcholine
DPH - 1,6-diphenyl-1,3,5-hexatriene
SFA - saturated fatty acid
UFA - unsaturated fatty acid

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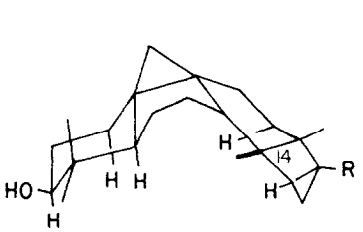
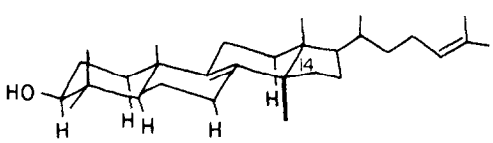
selective demethylation of lanosterol at C_{14} and C_4. The dealkylated planar α-face of cholesterol appears to be optimal for membrane function (3), e.g. reduction of glucose permeability or increase of the microviscosity of lecithin vesicles (4). We now show that cycloartenol, a 9,19-cyclopropane plant sterol (I) which is isomeric with lanosterol (II) condenses fatty acyl chains of phospholipids more effectively than lanosterol both in artificial and natural membranes.



(I) R′–

(III) R″–



(II)

The methyl groups at C-14 are indicated by heavy bars.

We also show that the related 9,19-cyclopropane 24-methyl sterol, cyclolaudenol (III) does not raise the microviscosity of either artificial or natural membranes and that its effectiveness as a growth factor for M. capricolum is comparable to that of lanosterol.

MATERIALS AND METHODS. Chemicals. Cholesterol (Sigma) was recrystallized from ethanol and dried in vacuo. Lanosterol (Sigma) was purified according to established procedures (5). Cycloartenol and cycloartanol were gifts of D. Arigoni. Cyclolaudenol was a gift from G. Ourisson. All sterols were 99% pure by gas-liquid chromatography. Heptadecanoic acid, elaidic acid, and 5α-cholestane were from Sigma, palmitic acid from Supelco, and egg yolk phosphatidylcholine (Ptd Cho) from Avanti.

Growth of M. capricolum and isolation of membranes. M. capricolum (California kid strain 14, ATCC 27342) was cultured on a modified delipidated Edward medium (6). The PPLO-serum fraction was replaced by 1 mg/ml fatty acid free bovine serum albumin (fraction V, Sigma), 10 µg/ml sterol, 5 µg/ml palmitate, and 6.5 µg/ml elaidate. Growth was followed spectrophotometrically

by reading absorbances at 640 nm. Cells were harvested in the late logarithmic phase of growth and membranes isolated and prepared for lipid extraction and microviscosity studies as described (6). Membrane protein was determined by the procedure of Lowry et al. (7).

Lipid analysis of M. capricolum membranes. Membrane lipids were extracted (8), saponified, and separated into non-saponifiable and fatty acid fractions. Fatty acid methyl esters were generated with 14% boron trifluoride in methanol. Lipids were quantitated by gas-liquid chromatography on a Perkin-Elmer instrument (model 900) equipped with a 1.8 m column of 3% SP2250 (Supelco) at 260°C for sterols and 10% SP2330 (Supelco) for fatty acid methyl esters with a temperature program from 150° to 210°C increasing at 6°C/min.

Vesicle Preparation. Vesicles containing test sterols were prepared by the procedure of Szoka and Papahadjopoulas (9). For sterol analysis the phospholipid vesicles were passed through a Sepharose 4B column to remove incorporated sterol. Sterol and phospholipid in the eluted vesicles when quantitated by gas-liquid chromatography were found to be present in the ratios added originally.

Microviscosity measurements of vesicles and mycoplasma membranes. Microviscosities were determined in an Elscint microviscosimeter, Model MV-1A, using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe (10,11). To 1 ml of the vesicle preparation (1.29 µmol in Ptd Cho) or 1 ml of mycoplasma membranes (1 mg protein/ml) was added 1 ml of a 1 µM solution of DPH in 10 mM NaCl. This mixture was incubated at 37°C for 30 min. Fluorescence depolarization of DPH was then measured at 25°C for Ptd Cho vesicles and 37°C for mycoplasma membranes, and microviscosities calculated according to Shinitzky and Inbar (12).

RESULTS. Microviscosities of model membranes containing cyclopropane sterols. The results of microviscosity determinations using the fluorescent probe DPH are given in Figure 1. At all concentrations, but especially above 30 mol %, cycloartenol and cycloartanol (24,25-dihydrocycloartenol) increased the microviscosity of Ptd Cho vesicles to a much greater extent than lanosterol. On the other hand, cyclolaudenol, the 9,19-cyclopropane sterol carrying an extra methyl group at C_{24} affected microviscosity to the same minimal degree as lanosterol.

Growth of M. capricolum on various cyclopropane sterols. The same cyclopropane sterols (Figure 1) were tested as growth supplements for the sterol requiring mycoplasma strain. The positive growth response of this organism to cycloartenol has already been reported (13).

The relative efficiencies of cholesterol, cycloartenol, and lanosterol as growth factors are shown in Figure 2. In the absence of any sterol supplement measurable growth did not occur. Cycloartenol and cycloartanol produced identical growth responses relative to cholesterol and lanosterol.

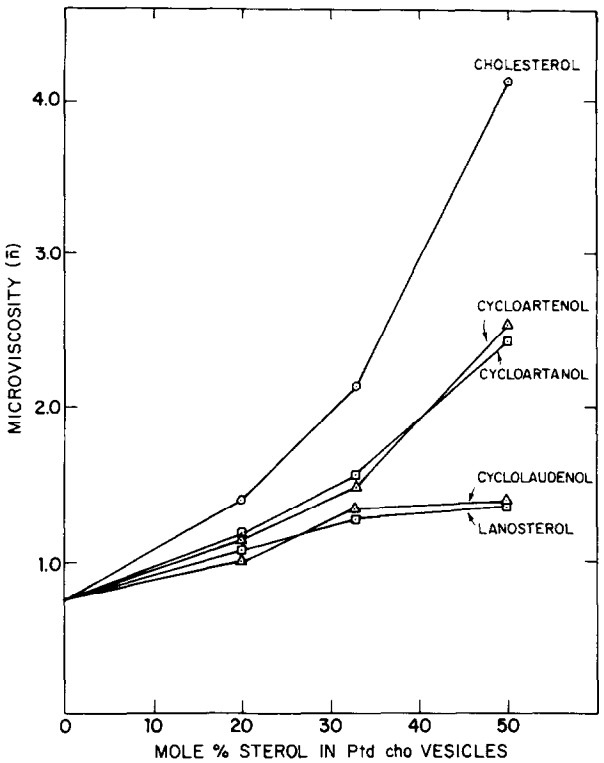


Figure 1 The effect of sterol structure on the microviscosity ($\bar{\eta}$) of Ptd Cho vesicles at 25°C.

With cyclolaudenol growth was slower than with cycloartenol and comparable to lanosterol. Examination of Figures 1 and 2 reveals a remarkably close correlation between the ability of the 9,19-cyclopropane sterols to increase

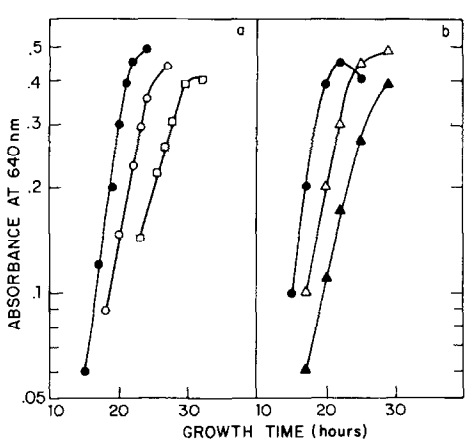


Figure 2 Growth of M. capricolum at 37°C on (a) cholesterol, ●; cycloartenol, ○; or lanosterol, □; (b) cholesterol, ●; cycloartanol, △; or cyclolaudenol, ▲.

TABLE I. The Effect of Sterol Structure on Sterol Content, Fatty Acid Distribution, and Microviscosity of M. capricolum Membranes[a]

Sterol added to growth media	Mole % sterol of total lipid	SFA/UFA[b]	P Value	Micro-viscosity ($\bar{\eta}$)
Cholesterol	31.7	0.84	0.305	4.77
Cycloartenol	35.1	0.85	0.295	4.31
Cycloartanol	29.5	0.89	0.290	4.09
Cyclolaudenol	20.0	0.91	0.240	3.34
Lanosterol	29.3	0.83	0.265	3.16

[a]Cells were cultured on lipid depleted modified Edward medium as described in the Materials and Methods section.

[b]The fatty acid species added accounted for > 97% of those found in the membrane phospholipids.

the microviscosity of artificial membranes and their efficacy as growth factors of M. capricolum. A given structural modification changes both parameters in the same direction.

Lipid composition and microviscosity of M. capricolum membranes. Fatty acid composition, sterol content and the microviscosities of the membrane fractions isolated from each of the sterol-grown cultures are given in Table I. The sterol content of isolated membranes ranged from 20 to 35 mol %. There is no correlation between sterol content per se and the rate of growth. For example, cycloartenol showed a higher enrichment than cholesterol, yet it supported growth somewhat more poorly (Figure 2). Similarly, whereas M. capricolum grew at approximately the same rate on lanosterol and cyclolaudenol, the cellular sterol content was higher with the former than with the latter. Growth rates are therefore not a direct function of total sterol incorporation.

Closely related with mycoplasma growth rates are the microviscosities of the isolated cell membranes (Table I). The more efficient a sterol as a growth factor, the greater the microviscosity ($\bar{\eta}$) values of the isolated cell

membranes. Sterol structure appears to determine growth rates in proportion to its ability to raise the microviscosity of the receptor membrane. The ratios of saturated to unsaturated fatty acid remained relatively constant in cells grown on the various test sterols.

DISCUSSION. Incorporation and localization of cholesterol in phospholipid bilayers appears to be a consequence of the extended hydrophobic overlap and hence contacts between the hydrocarbon chains of phospholipid and the planar ring system of cholesterol. Therefore, the measurable effects of cholesterol on phospholipid bilayers can be attributed to differential restriction of the number of rotational isomers permissible for different segments of the fatty acyl chains. However, the critical contact regions of the sterol molecule are still poorly defined. That the 14α-methyl group of lanosterol interferes markedly with sterol-phospholipid acyl chain interactions has previously been demonstrated (4,14). In the present study we show that cycloartenol, an isomer of lanosterol, is substantially more competent as a membrane sterol even though it contains, like lanosterol, an axial 14α-methyl substituent. As we now show, the deleterious effect of the 14α-methyl group can be eliminated not only by physical removal but also by changing the angular orientation of this substituent.

Cycloartenol, the product of plant squalene epoxide cyclases (15,16), is isomeric with lanosterol. It contains a 9,19-cyclopropane ring instead of the angular methyl group at C-10, but it is otherwise identical with lanosterol. Stereomodels reveal however remarkable conformational differences between the two molecules. In lanosterol the 14α-methyl group projects from the otherwise planar α-face. In contrast, the sterol ring system of cycloartenol is bent, forced into a non-planar conformation by the 9,19-cyclopropyl group on the β-face of the molecule[1]. In this bent conformation the

[1]We are aware of a recent report by Allen and Trotter (17) showing that the X-ray structure of the cycloartenol derivative cyclograndisolide indicates the C ring to be 1,3-diplanar (i.e. ring C has a conformation between a boat and a sofa).

sterol α-face is curvilinear instead of planar with the consequence that the 14α-methyl group is no longer exposed. Instead of protruding from the α-face it becomes embedded in an arc or belt of several axial α-face H-atoms. On the basis of the conformation revealed by Dreiding stereomodels we therefore postulate that the 14α-methyl group of cycloartenol unlike that of lanosterol promotes or at least does not interfere with sterol-phospholipid fatty acyl chain contacts. For these conformational reasons cycloartenol is competent for membrane function in spite of the non-planarity of the α-face. Similar arguments apply to the results obtained with a yeast auxotroph reported in the accompanying paper (18).

We would like to propose that the respective conformations of lanosterol and cycloartenol also determine the further metabolic fates of the two sterol precursors. In the lanosterol-cholesterol pathway in mammalian and yeast cells the methyl group at C-14 is the first of three methyl groups to be removed, affording $\Delta^{8(9),14}$-4,4-dimethylcholestadienol (19,20). In photosynthetic organisms cycloartenol demethylation takes a different course, as Heintz and Benveniste have shown (21). A cell-free enzyme system from bramble tissue (Rubus fructicosus) grown in culture metabolizes cycloartenol to a 4α,14α-dimethyl derivative (cycloeucalenol) by removal of the 4β-methyl group prior to metabolic demethylation at C-14.

We suggest that in the bent cycloartenol conformation the buried C-14 methyl group is not only neutral or even beneficial for membrane function but may also be inaccessible to enzymatic attack. The priorities and sequence for methyl group removal therefore change. After removal of the axial 4β-methyl group the cyclopropyl group is opened by enzyme-mediated proton attack as Heintz and Benveniste have shown (21). The resulting obtusifoliol (4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol) assumes the "normal" planar conformation with an axial methyl group at C-14 that is exposed as in lanosterol and therefore accessible to oxidative enzymes.

Unlike cycloartenol, cyclolaudenol which contains an added methyl group at C-24 in the isooctenyl side chain does not increase the microviscosity of model membranes, effectively promote the growth of M. capricolum, or increase the microviscosity of M. capricolum membranes. Alkyl substitution at C-24 of the side chain is unique to sterols in plants and fungi (22). Our results showing that the presence of side chain alkyl substitution reduces membrane competence, i.e. weakens fatty acyl chain-sterol interactions, suggests that alkylated sterols may play a role in plant and fungal membranes that differs, at least in detail, from the role of cholesterol in mammalian cell and mycoplasma membranes.

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