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The perturbing effect of cholesterol on the interaction between labdanes and DPPC bilayers

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Abstract

Differential scanning calorimetry (DSC) was used to study the effect of cholesterol on the perturbation of DPPC bilayers induced by eight bioactive structurally related labdanes isolated from the resin 'ladano' of *Cistus creticus* subsp. *creticus* (Cistaceae) or semisynthesized from the mother compounds. Labdanes themselves induced profound modifications in DPPC bilayer organization and thermotropic properties that were altered when cholesterol was incorporated in equimolar amounts to the labdanes. The present work shows that, up to 10 mol% of the equimolar mixture of cholesterol and the labdanes, the modifications evoked on DPPC bilayer organization are in accordance to these induced by the labdanes themselves. When the concentration exceeded 20 mol%, cholesterol influence dominated while the effect of the labdanes was suppressed and their interaction with the bilayer was probably prevented. The degree by which cholesterol modulated the labdane interaction with the bilayer depended on their structural characteristics that determine their localization in the bilayer interior. Polar groups that force the labdanes to localize themselves at the interfacial region broadened the concentration range by which labdanes interacted with the DPPC bilayer even in the presence of high concentration of cholesterol where cholesterol-rich domains are preferentially formed. On the other hand, labdanes possessing functional groups that promote their deeper penetration in the bilayer interior compete with cholesterol in a high extent for the same localization sites resulting in their possible elimination from the bilayer when the concentration of cholesterol present exceeds the 20 mol%.
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Keywords: Labdanes; Cholesterol; DSC; DPPC bilayer; Localization competition

1. Introduction

Labdane type diterpenes represent a significant class of natural products inducing a variety of biological responses. Among others, labdanes isolated as major components from the resin 'ladano' of *Cistus creticus* subsp. *creticus* (Cistaceae) have proved to be *in vitro* active antimicrobial agents and cytotoxic agents against human leukemic and solid tumor cell lines [1–8]. Labdanes **1** and **3**–**6** are the major components of the resin 'ladano', while labdane **2** and sclareol are minor constituents and labdane **7** a semisynthetic derivative of labdane **5** (Fig. 1).

Although the mechanism by which the specific labdanes exhibit their biological activity are not yet clearly understood, recent studies suggested the induction of apoptotic cell death in human T leukemic cells, interfering with their cell cycle, arresting cells at $G_{0/1}$ phase *via* a *p53* independent pathway [6,9]. Those labdanes possessing both a hydrophobic carbon skeleton and oxygenated functions at several positions are amphiphilic water-insoluble compounds. Their hydrophobicity makes them able to interact with matrices of phospholipids. The pharmacological activity of amphiphilic drugs or bioactive molecules may be associated to their ability of interacting with the phospholipid matrix of the cell membrane [10,11]. Phosphatidylcholines have been studied extensively as a simple model of the cell membrane in the absence or presence of additives [12–17]. DSC is a sensitive tool in the exploration of lipid phase transitions and the elucidation of the effect of drugs on the dynamic properties of membrane components. However, the majority of the studies involving drug–membrane interactions do not include cholesterol, which account for nearly 50% of the total lipids in some cases. Cholesterol has been assumed to alter packing of

Abbreviations: Labdane **1**, labd-13(*E*)-ene-8α,15-diol; Labdane **2**, labd-13(*E*)-ene-8α-ol-15-yl-acetate; Labdane **3**, labd-7,13-dien-15-ol; Labdane **4**, labd-7,13-dien-15-yl-acetate; Labdane **5**, 3β-hydroxy-manoyl oxide; Labdane **6**, 3β-acetoxy-manoyl oxide; Labdane **7**, *N*-imidazole-3β-thiocarbonyl-ester of manoyl oxide; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry

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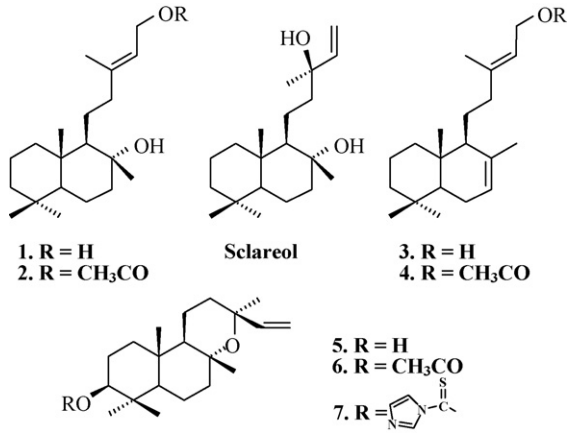

Fig. 1. Chemical structures of labd-13(*E*)-ene-8α,15-diol (labdane **1**), labd-13(*E*)-ene-8α-ol-15-yl-acetate (labdane **2**), sclareol, labd-7,13-dien-15-ol (labdane **3**), labd-7,13-dien-15-yl-acetate (labdane **4**), 3β-hydroxy-manoyl oxide (labdane **5**), 3β-acetoxy-manoyl oxide (labdane **6**) and *N*-imidazole-3β-thiocarbonyl-ester of manoyl oxide (labdane **7**).

the phospholipids giving rise to overlapping sharp and broad transitions due to lateral phase separation into cholesterol-poor and rich domains, respectively. The sharp components of the main transition of DPPC bilayers disappear as the concentration of cholesterol rises above 20 mol% [18–22]. Cholesterol may hinder intercalation of additives into the hydrophobic core of the bilayer when its concentration exceeds 20 mol% [23–25]. In previous work the interaction of labdanes with DPPC bilayers was investigated by DSC [8,26,27]. The labdanes was found to strongly affect the bilayer structural organization and induce changes in the thermotropic behaviour of DPPC in a concentration dependent manner. These changes include elimination of the pretransition peak, appearance of new low enthalpy peaks below the main transition of DPPC, the growth of low temperature peaks at the expense of the main transition of DPPC in some cases, decrease in the cooperativity of the transition, and induction of mixed phases. The present work provides information on the influence of cholesterol on the thermotropic changes induced by the presence of labdanes.

2. Materials and methods

2.1. Materials

Labdanes **1**, **3**–**6** were isolated from the resin 'Ladano' of the plant *C. creticus* subsp. *creticus* that grows in the island of Crete (Greece) (collector Dr. C. Demetzos). Column chromatography for isolating and purifying the labdanes was performed on silica gel (Merck, 230–400 mesh). Analytical and preparative thin layer chromatography was carried out on Merck silica gel F_{254} plates. *N*,*N*′-Thiocarbonyl-diimidazole was purchased from Fluka (Fluka Chemie GmbH). DPPC was obtained from Avanti Polar Lipids Inc. (Albaster, AL, USA). Cholesterol and sclareol were obtained from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA). Solvents of analytical grade were obtained from Labscan Ltd. HPLC grade water was obtained by PRO^{TM} PS Labconco System.

2.2. Methods

2.2.1. Isolation of labdanes

The 4.1 g of the CH_2Cl_2 extract of the resin "ladano" were fractionated by column chromatography using as eluents cyclohexane, cyclohexane/EtOAc mixtures of increasing polarity. Fractions obtained were further fractionated by column chromatography using as eluents, volumes of *n*-hexane/EtOAc 99:1 and *n*-hexane/EtOAc 98:2, respectively. Labdane **1** was isolated as white solid [$\alpha_D = +0.2^\circ$ (*c* 0.35 mg/ml $CHCl_3$)], labdanes **3** and **4** as yellow oils [$\alpha_D = +5.6^\circ$ (*c* 0.67 mg/ml $CHCl_3$) and $\alpha_D = +6.2^\circ$ (*c* 0.50 mg/ml $CHCl_3$), respectively], and labdanes **5** and **6** as white solids [$\alpha_D = -23.1^\circ$ (*c* 0.65 mg/ml $CHCl_3$) and $\alpha_D = -60.6^\circ$ (*c* 0.60 mg/ml $CHCl_3$), respectively]. Structure elucidation was performed on a NMR 400 MHz Bruker spectrometer. The purity of the labdanes was 99.5% (**1**), 95.9% (**3**), 95.8% (**4**), 100% (**5**) and 99.6% (**6**), respectively, as determined by GC–MS [Hewlett Packard (HP) 5973 mass selective detector, and a DB-5 Ms fused silica capillary column of a 30 m × 0.25 mm (0.25 μm film thickness)].

2.2.2. Chemical transformation of labdane *1* to its acetylated derivative and of labdane *5* to its N-imidazole-thiocarbonyl-ester

The 20 mg of labdane **1** were treated with a volume of 4 ml acetic anhydride in pyridine (1:1 v/v) for at least 2 h in dark at room temperature to yield a yellow oil of labdane **2** [$\alpha_D = +3.3^\circ$ (*c* 0.70 mg/ml $CHCl_3$)]. Labdane **2** was purified over a silica gel column eluted with CH_2Cl_2. Structure elucidation was performed on a NMR 400 MHz Bruker spectrometer. The purity of labdane **2** was 99.4% as determined by GC–MS.

Labdane **5** was treated with *N*,*N*′-thiocarbonyl-diimidazole in CH_2Cl_2 at 1:2 molar ratio at least for an hour before their solution was subjected under reduced pressure (60 mm Hg) for an additional hour. When needed the solvent was renewed. The excess of the reagents was removed by hexane precipitation and labdane **7** was purified by preparative TLC [$\alpha_D = -45.9^\circ$ (*c* 0.60 mg/ml $CHCl_3$)]. Structure elucidation was performed on a NMR 400 MHz Bruker spectrometer. The purity of labdane **7** was 97.7% as determined by HPTLC-FID (high performance thin layer chromatography coupled to a flame ionization detector, Iatroscan MK-5^{new}, Iatron Lab. Inc., Tokyo, Japan instrument) according to the literature [8].

2.2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed on an 822^e Mettler-Toledo (Schwerzenbach, Switzerland) calorimeter calibrated with pure indium ($T_m = 156.6\,^\circ C$). Sealed aluminum crucibles having 40 μl capacity were used as sample holders. The sample scan rate was 2 °C/min on heating and 20 °C/min on cooling and thermograms were obtained between 10 and 60 °C. Enthalpy changes and characteristic transition temperatures were calculated with Mettler-Toledo STAR^e software. Appropriate amounts of DPPC and cholesterol or equimolar mixtures of the labdanes and cholesterol were dissolved in chloroform and the solvent was removed by gentle bubbling of dry nitrogen gas. The residual solvent was removed

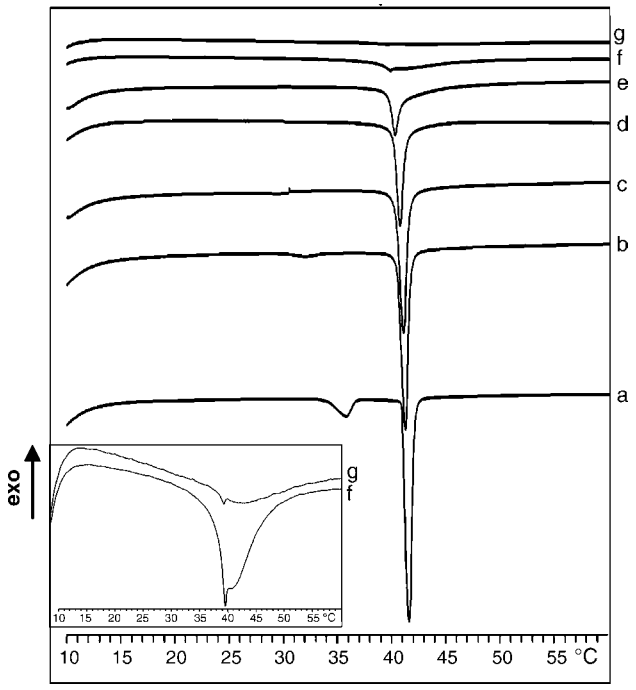

Fig. 2. DSC heating thermograms normalized to sample weight of fully hydrated DPPC bilayers in the presence of cholesterol at concentrations of (a) 0 mol%, (b) 2.5 mol%, (c) 5 mol%, (d) 10 mol%, (e) 15 mol%, (f) 20 mol% and (g) 30 mol%. Enlargement of the thermograms in the presence of high concentration of cholesterol are presented so phase separation becomes visible.

under vacuum overnight. The mol% of cholesterol and the equimolar mixture of cholesterol and the labdanes in DPPC bilayers were 2.5, 5, 10, 15, 20 and 30%, respectively. The dry material was weighted into the aluminum crucibles, and HPLC-grade H_2O was added at 50 wt%. Prior to measurements the crucibles were subjected to a temperature over the transition of DPPC to ensure equilibration. All samples were scanned repeatedly until identical thermograms were obtained. An empty aluminum crucible was used as reference.

3. Results

DSC profiles obtained for pure DPPC and mixtures of DPPC with cholesterol or equimolar mixture of cholesterol and the labdanes are shown in Figs. 2–4, and the calorimetric parameters (T_m, ΔH and $\Delta T_{1/2}$) are summarized in Fig. 5. In the absence of any additive, DPPC exhibits a pretransition event centered at 36.1 °C accompanied by a small transition enthalpy of 5.3 kJ/mol from the lamellar gel phase L'_β to the rippled gel phase P'_β. The main transition event was at 41.7 °C with an enthalpy of 35.4 kJ/mol from the P'_β phase to the liquid crystalline phase L_α [28]. The cholesterol-induced thermotropic modifications are in accord with literature [18,19,21,22] (Fig. 2). In all cases, the presence of equimolar cholesterol/labdane mixtures strongly affected the pretransition event. A low concentration of the equimolar mixture shifted the pretransition temperature to lower temperatures and decreased the enthalpy, while a concentration of 2.5–5 mol% eliminated the pretransition peak. The disappearance of the pretransition event is caused by binding of the additives to the head group region. Cholesterol is known to anchor its 3β-hydroxyl group in the interface region leaving its hydrophobic carbon ring system to penetrate the acyl chain region [21,29]. The labdanes also possess at least one hydroxyl group able to interact with the carbonyl group of the ester bond thus affecting the structure of the head group region. That orientation would potentially reduce head group crowding in the interfacial region and the cross-sectional area mismatching with the acyl chains that is postulated to be responsible for the appearance of the ripple phase in phosphatidylcholine bilayers [30,31]. Thus, small amounts of equimolar mixtures of cholesterol and the labdanes could either stabilize, as for cholesterol alone [31–34], or eliminate the ripple phase, but in either case the pretransition peak would gradually disappear.

The presence of increasing concentrations of the equimolar cholesterol/labdane mixtures caused a gradually reduction of the main transition temperature T_m (Fig. 5A), as a result of the hydrophobic mismatching between the interactive molecules (i.e. acyl chains and labdanes) that enhances *trans–gauche* transformations and is related to a bilayer fluidization effect. The T_m reduction depended on the structural features of each labdane that may influence the orientation and positioning of them in the bilayer interior. Based on the enthalpy change (ΔH) during the main transition, the presence of increasing concentration of any cholesterol/labdane mixture intercalating between the phospholipid acyl chains affected significantly the relative stability of gel and liquid crystalline phases implying that the penetration of both molecules in the bilayer hydrophobic interior may cause an interchain connectivity disturbance. A slight increase was observed in the concentration range of 2.5–5 mol% followed by a drastic reduction in accord to that caused by the presence of cholesterol itself (Fig. 5B). It seems likely that the labdanes at low concentration are able to induce gel phase stabilization as long as cholesterol concentration is kept low, while at higher concentration cholesterol's gel phase destabilization effect dominate and that may reflect a reduced penetration of the labdanes in the bilayer interior. In the presence of increasing concentrations of the equimolar cholesterol/labdane mixture the main transition peak gradually broadened with increasing asymmetry. Significant loss in the cooperativity of the transition, as indicated from the $\Delta T_{1/2}$ values (Fig. 5C), was caused in the presence of 10 mol% and more at which phase separation occurred, suggesting the induction of disorder in the lipid bilayer and the appearance of destabilized bilayer regions. Bilayer destabilization may have been caused by clustering of labdane molecules in separated domains interfering with the interchain connectivity and implies immiscibility with cholesterol giving reason for the higher than expected $\Delta T_{1/2}$ values in comparison to those induced by the labdanes or cholesterol themselves. In the case of labdane **2** and sclareol a new cooperative transition was restored at lower temperature as their concentration increased, as seen from the corresponding thermograms at Fig. 3B and C. That effect was however observed also in the absence of cholesterol [26] and is probably caused by clustering of labdanes in separated enriched domains and cannot be attributed to the presence of cholesterol itself.

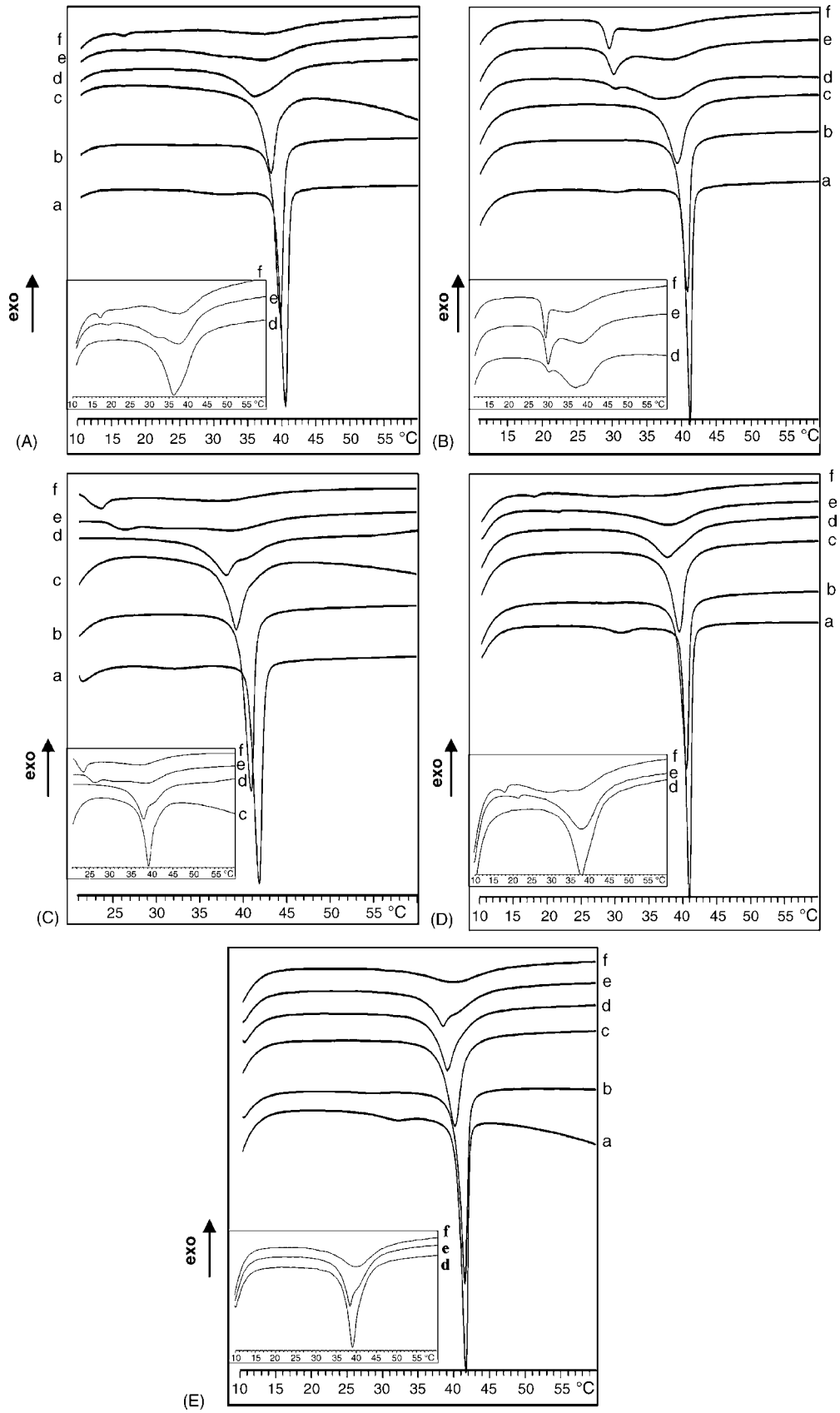

Fig. 3. DSC heating thermograms normalized to sample weight of fully hydrated DPPC bilayers in the presence of equimolar mixtures of cholesterol and (A) labdane **1**, (B) labdane **2**, (C) sclareol, (D) labdane **3** and (E) labdane **4** at concentrations of (a) 2.5 mol%, (b) 5 mol%, (c) 10 mol%, (d) 15 mol%, (e) 20 mol% and (f) 30 mol%. Enlargement of the thermograms in the presence of high concentration of the equimolar mixture of cholesterol and the labdanes are presented so phase separation becomes visible.

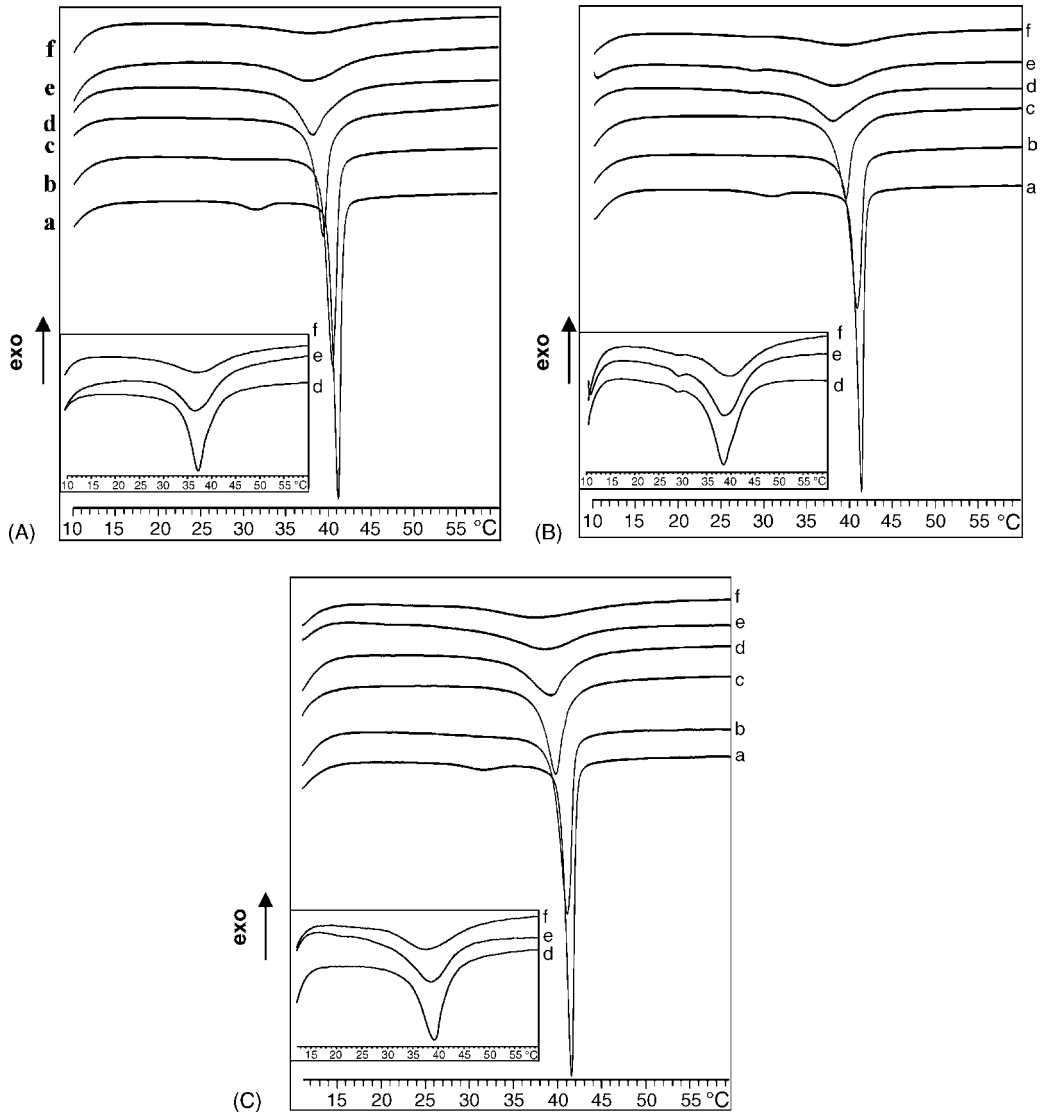

Fig. 4. DSC heating thermograms normalized to sample weight of fully hydrated DPPC bilayers in the presence of equimolar mixtures of cholesterol and (A) labdane **5**, (B) labdane **6** and (C) labdane **7** at concentrations of (a) 2.5 mol%, (b) 5 mol%, (c) 10 mol%, (d) 15 mol%, (e) 20 mol% and (f) 30 mol%. Enlargement of the thermograms in the presence of high concentration of the equimolar mixture of cholesterol and the labdanes are presented so phase separation becomes visible.

4. Discussion

It is known that at low cholesterol content, cholesterol-poor domains dominate in DPPC bilayers [19–22] providing thus sufficient space to labdanes for effective interaction with the bilayer. That is true in the presence of equimolar cholesterol/labdane mixtures at concentrations up to 10 mol%. At higher concentrations the thermodynamic changes resulting from the interaction of both are somehow dissimilar suggesting a key influence of cholesterol on the perturbing effect of the labdanes on DPPC bilayers. To ensure an easier comparison of the results, Fig. 6 reproduces the thermograms of DPPC containing 20 mol% of the labdanes and cholesterol themselves and their equimolar mixture at 30 mol%, at which each component correspond nearly to 20 mol% referred to DPPC. The influence of cholesterol on the perturbing effect of the labdanes is evident (dotted lines) as compared to the thermograms produced by the presence of these labdanes themselves (solid lines). Phase separation is still apparent in the thermograms however cholesterol modulates the interaction and probably the localization of the three labdanes in the lipid bilayer. The transition peaks revealed much broader than that observed when the labdanes themselves interacted with DPPC bilayers implying a significantly reduced cooperativity of the transition. In other words that broadening of the main transition peak represents the expansion in bilayer's density fluctuations resulting from the increased lateral heterogeneity and the coexistence of interacting or associated domains present within the bilayer [35]. The phases produced by domain formation may correspond either to interactions of labdanes with the bilayer since transition peaks were found nearly at same temperature as in the absence of cholesterol or to interactions of units produced by mixtures of labdanes and cholesterol as peak temperatures were found to occur at lower values (case of labdanes **1**, **2** and sclareol, Fig. 6A and B). As the con-

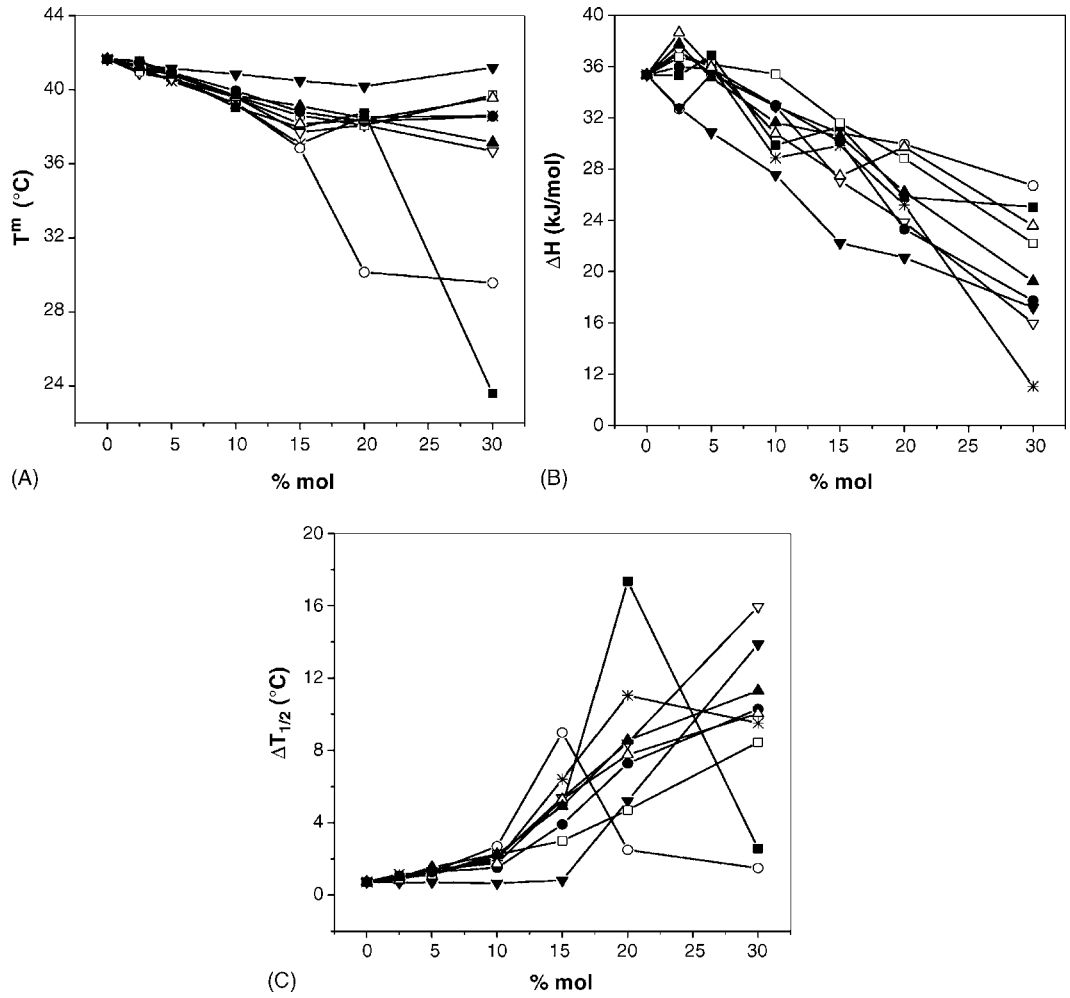

Fig. 5. (A) T_m (°C), (B) ΔH (kJ/mol) and (C) $\Delta T_{1/2}$ (°C) values of DPPC bilayers vs. concentrations of cholesterol (▼) and equimolar mixtures of cholesterol and labdane **1** (⁎), labdane **2** (◯), labdane **3** (▽), labdane **4** (□), labdane **5** (●), labdane **6** (△), labdane **7** (▲) and sclareol (■).

centration of individual the components reaches the 20 mol%, cholesterol-rich domains are expected to dominate in the bilayer as depicted in the corresponding thermogram (Fig. 6, curve a). Cholesterol-rich domains seem likely to modulate significantly the extent of interaction of the labdanes with the DPPC bilayer as a result of the competition for similar location sites. These domains virtually reducing the free volume for labdane incorporation and interaction emerged nearly inaccessible to the later. That effect is evident comparing the thermograms in the presence and absence of cholesterol at which the former appeared less complicated and multiple domains are converted to simpler. This observation further supports the idea that the labdanes interact preferentially with cholesterol-poor domains at which the influence of cholesterol is limited and free space is available. However, as the transition temperature of the broad component of the thermograms corresponding to cholesterol-rich domains was slightly reduced in the presence of labdanes **1**–**3**, **7** and sclareol, it appeared likely that limited interaction of the later with these domains occurred. The anchoring of these labdanes in the interfacial region of the bilayer would provide a reasonable explanation since in that case the labdanes would not intercalate deeply between the acyl chains and some molecules might still have been able to interact with cholesterol-rich domains. The perturbing effect of labdanes **4**–**7** revealed entirely dissimilar compared to that in the presence of cholesterol at equimolar amounts. As evident in Fig. 6C and D, curves c and d, respectively, when cholesterol exceeded 20 mol% with reference to DPPC, labdanes **4** and **7** displayed minor or no interaction with the bilayer. It seems likely that their interaction with the bilayer is restricted at cholesterol-poor domains, but they are still limited judging from the slight modifications observed in the thermograms. The sharp component in the thermograms produced by cholesterol-poor domains has disappeared and the labdanes seems to have no detectable effect on the broad component suggesting their exclusion from the bilayer. The presence of the hydrogen bonding acceptor acetoxy group besides the lipophilicity of labdane **4** may have been responsible for the deeper penetration of the molecule in the interior of the bilayer. On the other hand, labdanes **5**–**7** due to their shape could penetrate the acyl chains in a similar manner as cholesterol. In both cases labdanes would compete with cholesterol for the same positioning in the bilayer. However, interesting appeared the incident of the comparable influence of cholesterol on the perturbing effect of labdanes **5**–**7** regardless the substitution at

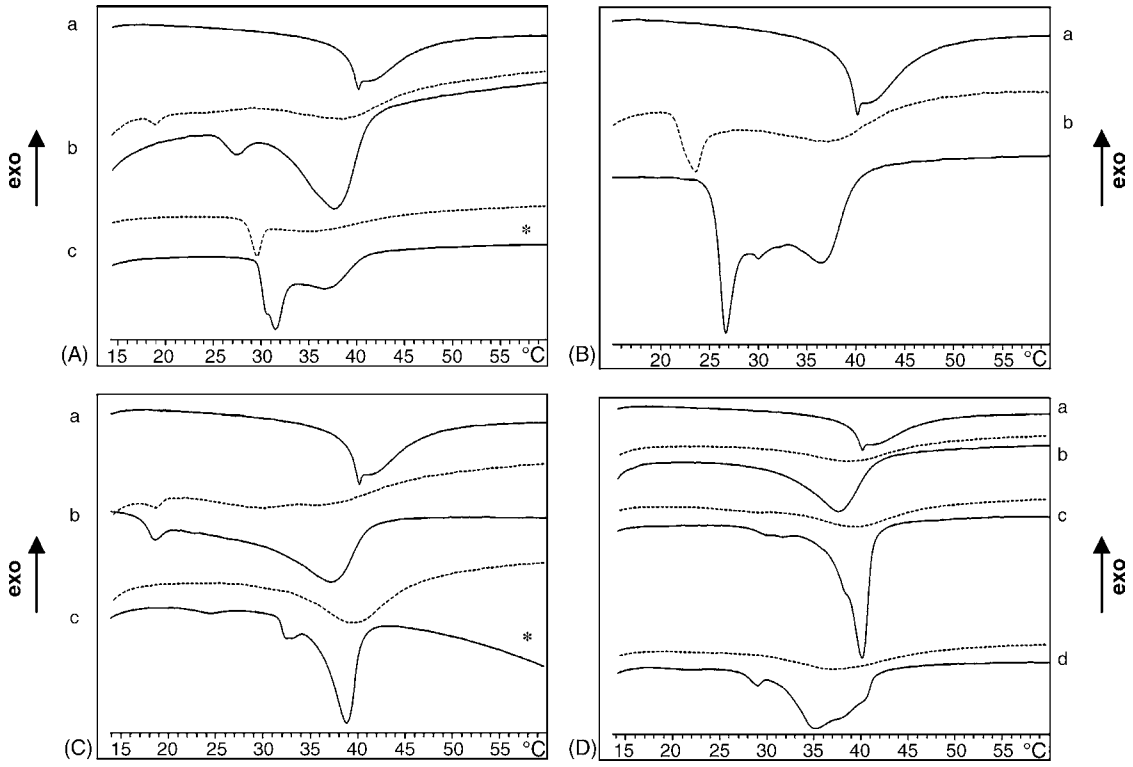

Fig. 6. DSC heating thermograms normalized to sample weight of fully hydrated DPPC bilayers in the presence (···) or absence (—) of cholesterol at 20 mol%. Equimolar cholesterol/labdane mixtures correspond to 30 mol%, but each components account for nearly the 20 mol% referred to DPPC. (A) (a) cholesterol, (b) labdane **1** and (c) labdane **2**; (B) (a) cholesterol and (b) sclareol; (C) (a) cholesterol, (b) labdane **3** and (c) labdane **4**; (D) (a) cholesterol, (b) labdane **5**, (c) labdane **6** and (d) labdane **7**. The endothermic flow values are divided by a factor of 2 (*) where indicated. Thermograms obtained in the absence of cholesterol were retrieved from Refs. [8,26,27].

3β-position. It appeared reasonable that the rigid carbon skeleton of labdanes **5**–**7** compete in any case with cholesterol partitioning in the bilayer interior as they share to a large extent similar structural features.

5. Conclusions

The thermotropic changes induced on DPPC bilayers as the concentration of the labdanes was increased in the presence of increased concentration of cholesterol was different from that observed by the presence of the labdanes themselves. The organization of the bilayer was affected in a similar way to that caused by the labdanes themselves as long as their equimolar mixture with cholesterol did not exceed 10 mol%, while it changed abruptly at higher concentrations where cholesterol modulated the effect of the labdanes on the bilayer. Labdanes possessing polar functional groups able to anchor at the interfacial region did not prevent interaction with the bilayer even at higher cholesterol concentrations; rather they were forced to occupy cholesterol-poor domains promoting lateral phase separation. Judging from the thermotropic changes induced by the presence and absence of cholesterol, labdanes partitioning deeper in the bilayer were excluded from this layer because high concentrations of cholesterol eliminate the free volume for the incorporation of labdanes in the DPPC bilayer. Cholesterol plays a key role in the degree of interaction of additives with lipid bilayers and such effects should be taken into consideration in any effort to correlate the thermotropic changes and bilayer structural modifications with the physiological activity of the additives.

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