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6. Peptide mimetics drugs and their interdigitation with lipid bilayers

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Abstract. AT_1 antagonists are a new generation of antihypertensive drug molecules known to exert their action in the transmembrane region of AT_1 receptors of lipid bilayers. Due to their amphipathic property AT_1 antagonists they are expected to act first in the lipid bilayer core and then to diffuse at the active site of the receptor. An approach of combining various physico chemical methodologies such as Differential Scanning Calorimetry, Raman and Solid State NMR Spectroscopy's are used to investigate their interdigitation effect into lipid bilayers. The obtained results point out that AT_1 antagonists are located in the interface of lipid bilayers where they exert strong interactions with head group and exert interdigitiation effect in the lipid bilayers. This effect may be related to their drug efficacy.

Introduction

There is considerable reported literature that lipid bilayers exert an interdigitation effect either alone or when a bioactive molecule is incorporated

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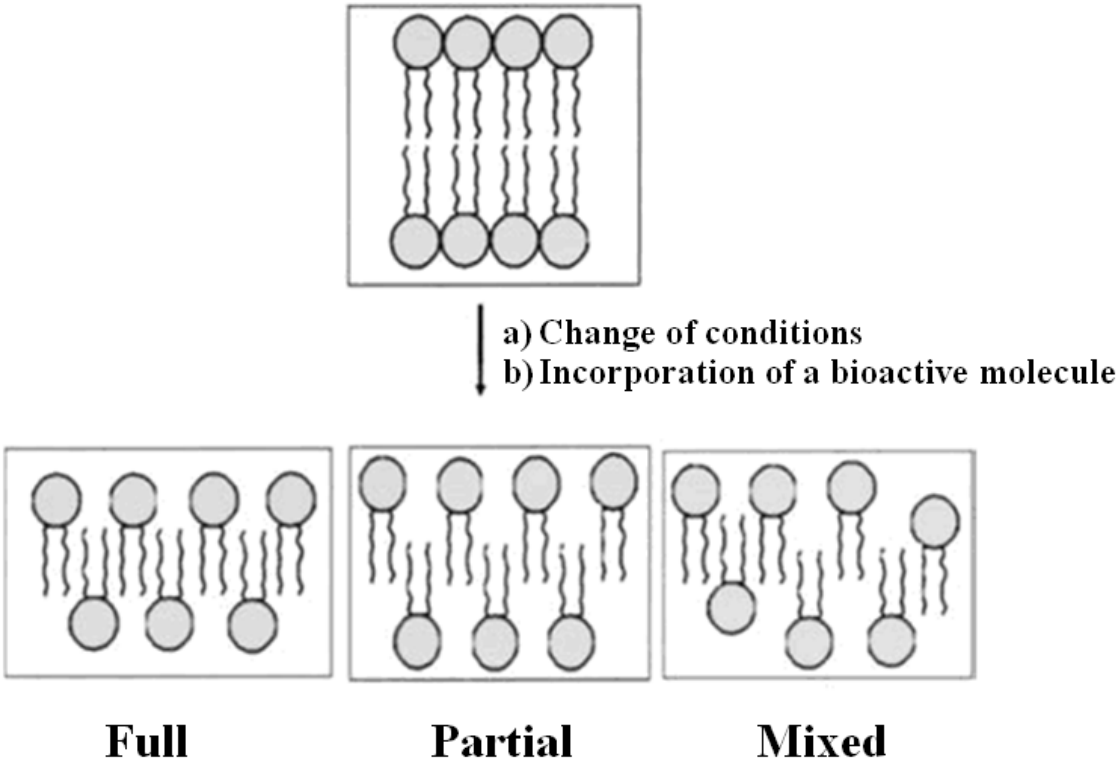

Figure 1. Various ways of inducing partial or total interdigitation.

within them (Figure 1). Thus, it is recorded that DHPC bilayers at normal conditions or 1,3 DPPC and DSPC bilayers at high pressure exert interdigitating effect.

The presence of a variety of chemical structures incorporated in lipid bilayers exert also interdigitating effect when they are incorporated in lipid bilayers. For example ethanol, benzyl alcohol, glycerol, vinblastine, tetracaine, labdanes, chlorpromazine etc are shown to induce interdigitating effect [1-10] (Figure 2).

The induction of interdigitation in the lipid bilayers is caused by molecules characterized as: (i) amphiphilic; (ii) preferably bulky; (iii) act on the interface; and (iv) not very long. Cholesterol is found to break the interdigitation effect since it is very long, comparable to the length of the lipid bilayers and it does not allow the alkyl chains of the two layers to interdigitate [8].

The conformational properties of ANG II and synthetic peptide analogs as well as commercially and synthetic AT_1 antagonists have been studied [11-12]. The prototype AT_1 antagonist losartan (COZAAR) was synthesized based on the mimicry of C-terminal segment of ANG II model proposed by Fermadjian *et al.* [13] (Figure 3).

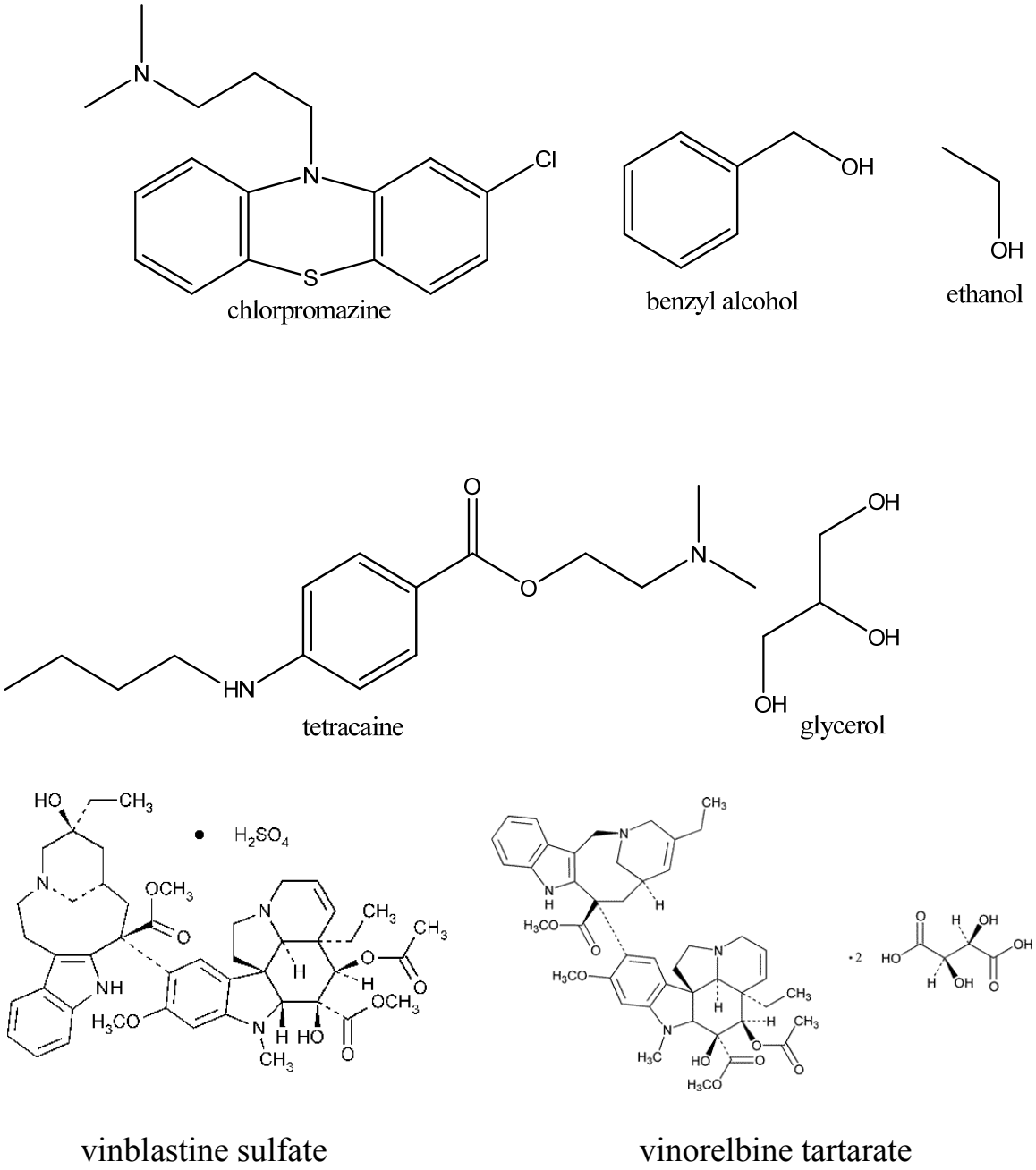

Figure 2. Reported molecules that induce interdigitating effect in lipid bilayers.

Angiotensin receptor blockers (ARBs) have been developed to produce a more complete blockade of the action of angiotensin II as compared to other drug classes, as well as an improved side effect profile [14-17]. The synthesis of losartan was followed by other SARTANS. In this review paper we will show some results for another molecule namely valsartan. Valsartan (Diovan) is the second orally active non-peptide angiotensin II specific to AT_1 receptor antagonist to reach the market in Europe and the USA for the treatment of hypertension. Several dose-findings and comparative studies have

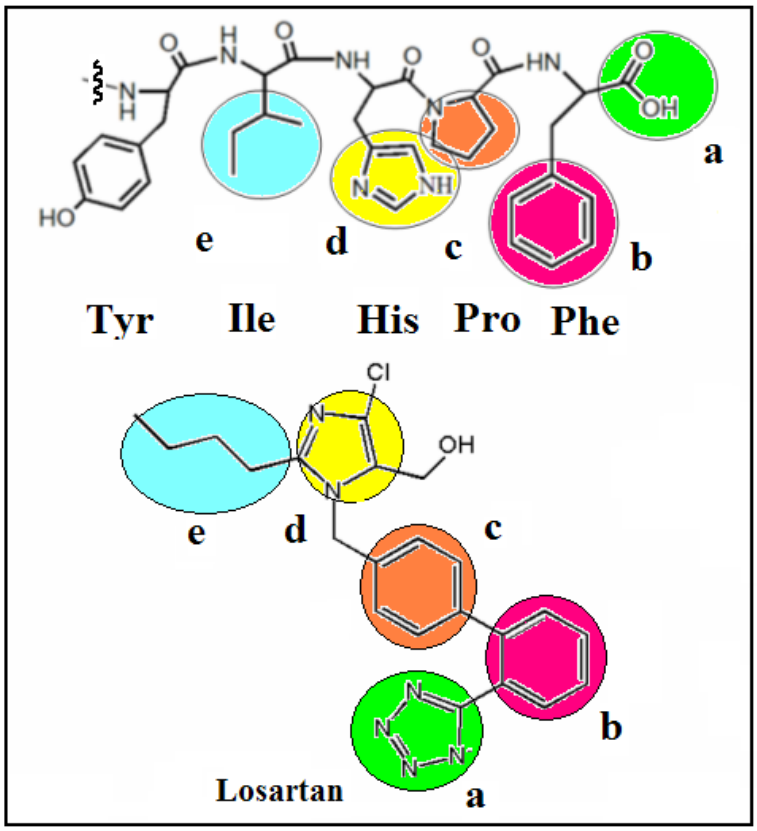

Figure 3. Mimicry of losartan with C-terminal segment of Angiotensin II. The letters show the corresponding similar molecular segments.

demonstrated that valsartan is an effective and well tolerated antihypertensive drug in patients with mild to moderate hypertension and to be effective in severe hypertension. Its effectiveness is at least equivalent to ACE inhibitors, diuretics, β-blockers and calcium antagonists and it has the advantage that does not cause cough and lower limb edema as ACE inhibitors [18-23]. Although, several drugs have been launched for the regulation of high blood pressure through the RAS cascade, intense research activity is still necessary to explore the structure requirements that direct to more effective antihypertensive drugs deprived of side effects. Due to the great need of more effective antihypertensive drugs, the collaborative laboratories have initialized a long term research activity for the Rational Design of novel AT_1 antagonists, mainly based on the conformational properties of marketed and synthetic molecules.

Based on superimposition studies of losartan with the model proposed for ANG II, a new avenue was explored in an attempt to design and synthesize novel AT_1 antagonists. Thus, few years ago we have briefly reported MMK1 synthesis, a simple molecule which possesses pyrrolidinone instead of biphenyltetrazole template. Its stereoelectronic properties were also analyzed

and compared with those of losartan [24,25]. MMK1 was designed to mimic conformational characteristics of His6-Pro7-Phe8 and constitutes the first lead compound. Two other derivative compounds were also synthesized namely MMK2 and MMK3. The structures of losartan, valsartan and MMK3 are shown in Figure 4.

Comparative *in vitro* binding studies with AT_1 and AT_2 receptors are performed, as well as *in vivo* experiments with adult normotensive male New Zealand white rabbits. All molecules were found to be less active than the prototype losartan.

Recently, we have proposed a mechanism of action of SARTANs at lipid bilayers. In this mechanism we have hypothesized that SARTANS like cannabinoids and calcium channel antagonists enter first the lipid bilayers and then diffuse to the active site of the receptor [26] (Figure 5).

Since the lipid bilayers constitute the first target to the receptor we have initiated a program to apply various physico chemical techniques to study AT_1 antagonists:lipid interactions and especially to examine if they cause interdigitation effect by acting on the interface.

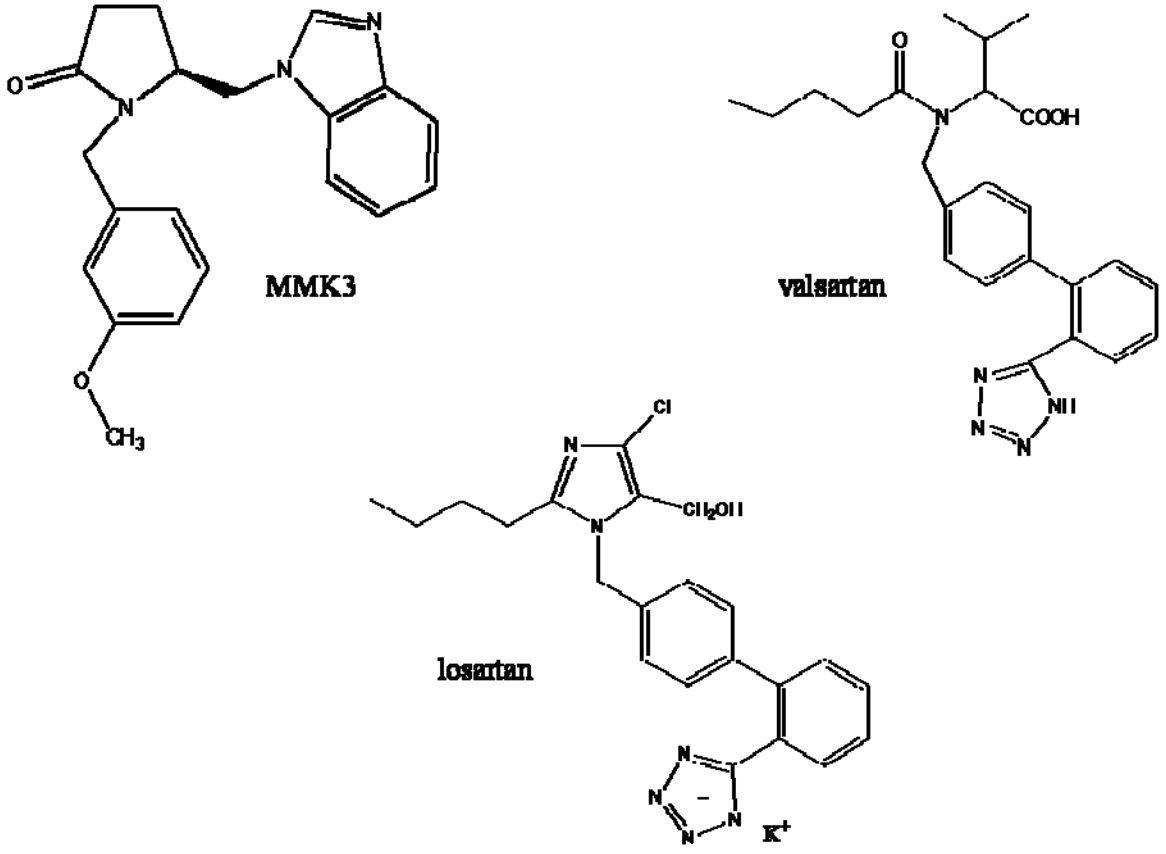

Figure 4. Structures of losartan, valsartan and MMK3.

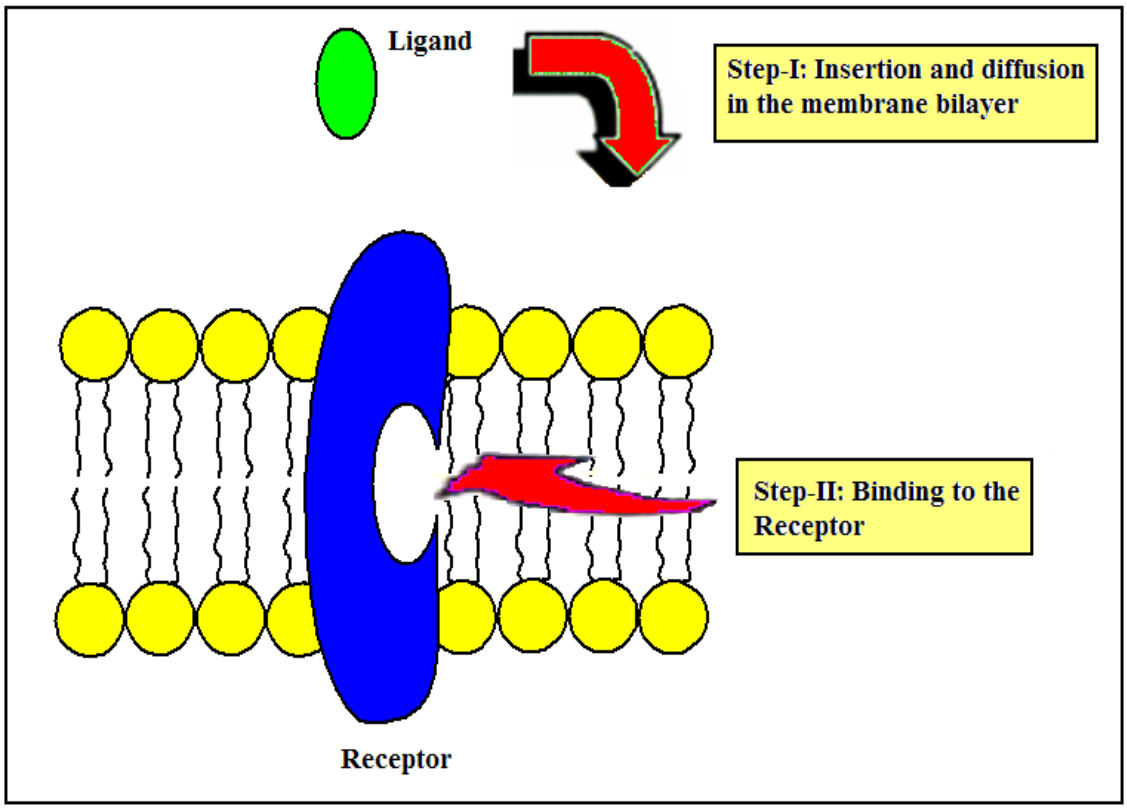

Figure 5. Molecular basis of SARTAN's action. The active drug is incorporated into the bilayer interface and laterally diffuses to reach the active site of the AT_1 receptor.

Results-discussion-conclusions

Molecular modeling

Molecular modeling studies [26] confirmed by ESR studies [27] show that losartan is located in the interface of the lipid bilayers with a high propensity of strong interactions with head-group (Figure 6). This localization as we mentioned above makes losartan a candidate for exerting interdigitation.

Therefore, it is of paramount importance to study their thermal and dynamic effects in lipid bilayers. For this reason, we have compared the commercial AT_1 antagonist valsartan with the synthetic analog MMK3, which possesses considerable lower activity. The synthesis of MMK3 is shown in Figure 7 [25]. To achieve this aim we have used two biophysical methodologies, namely, DSC and Raman spectroscopy.

Differential Scanning Calorimetry

Differential Scanning Calorimetry is a thermodynamic technique which is used extensively to study the thermal changes that drug causes in lipid

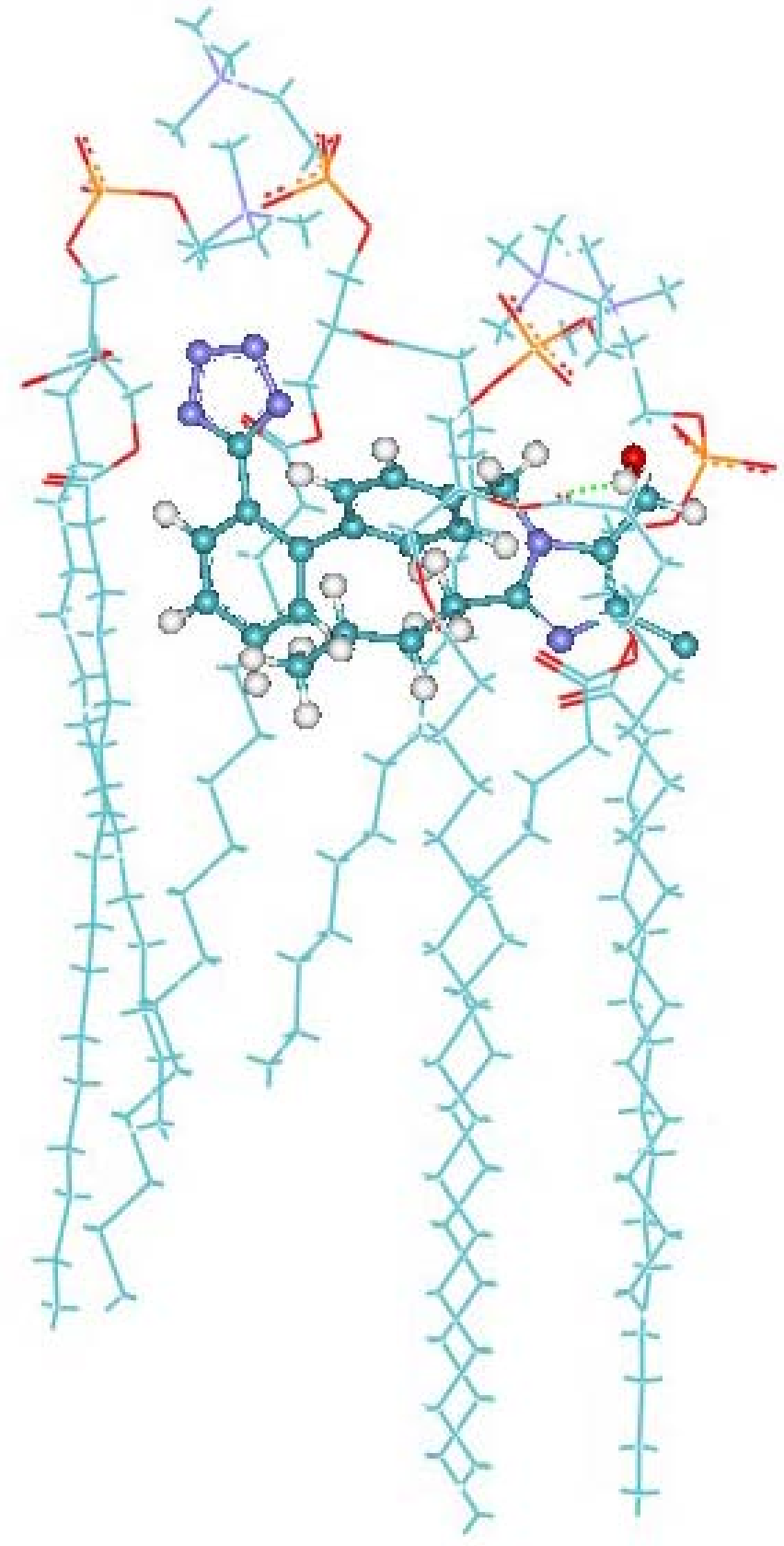

Figure 6. Localisation of losartan into lipid bilayers.

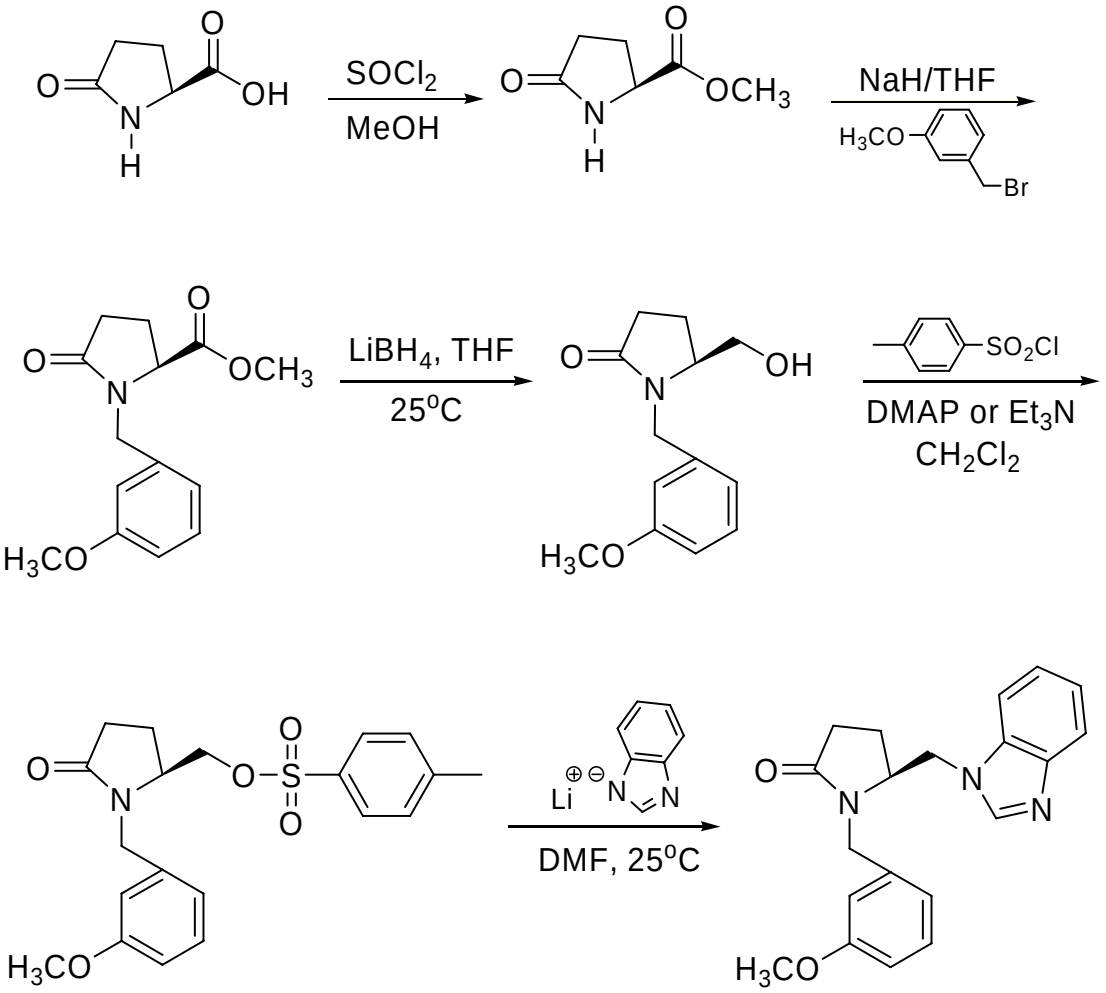

Figure 7. Synthesis of MMK3.

bilayers. DPPC bilayers are the most frequently used model bilayers, because some dynamic properties resemble those of biological membranes and because they show phase transitions at convenient temperatures. Hydrated DPPC lipids spontaneously form multi-lamellar bilayers whose *dynamic* and *thermotropic* properties have been extensively studied by various biophysical methods such as e.g. DSC and solid state Nuclear Magnetic Resonance (NMR) spectroscopy [28-32].

The DPPC molecules form at the lower temperatures a well organized lamellar gel phase, $L_{\beta'}$, while at high temperatures they obtain lamellar fluid phase, L_a which has greater fluidity. An intermediate phase, $P_{\beta'}$, is also observed, at which the bilayers are distorted by a *periodic ripple* (ripple phase). The DPPC bilayers exist in the $L_{\beta'}$ phase for temperatures lower than 35.3 ^{o}C and in the L_a phase for temperatures above 41.2 ^{o}C. The thermal changes caused by the inclusion of valsartan or MMK3 are shown in Figure 8.

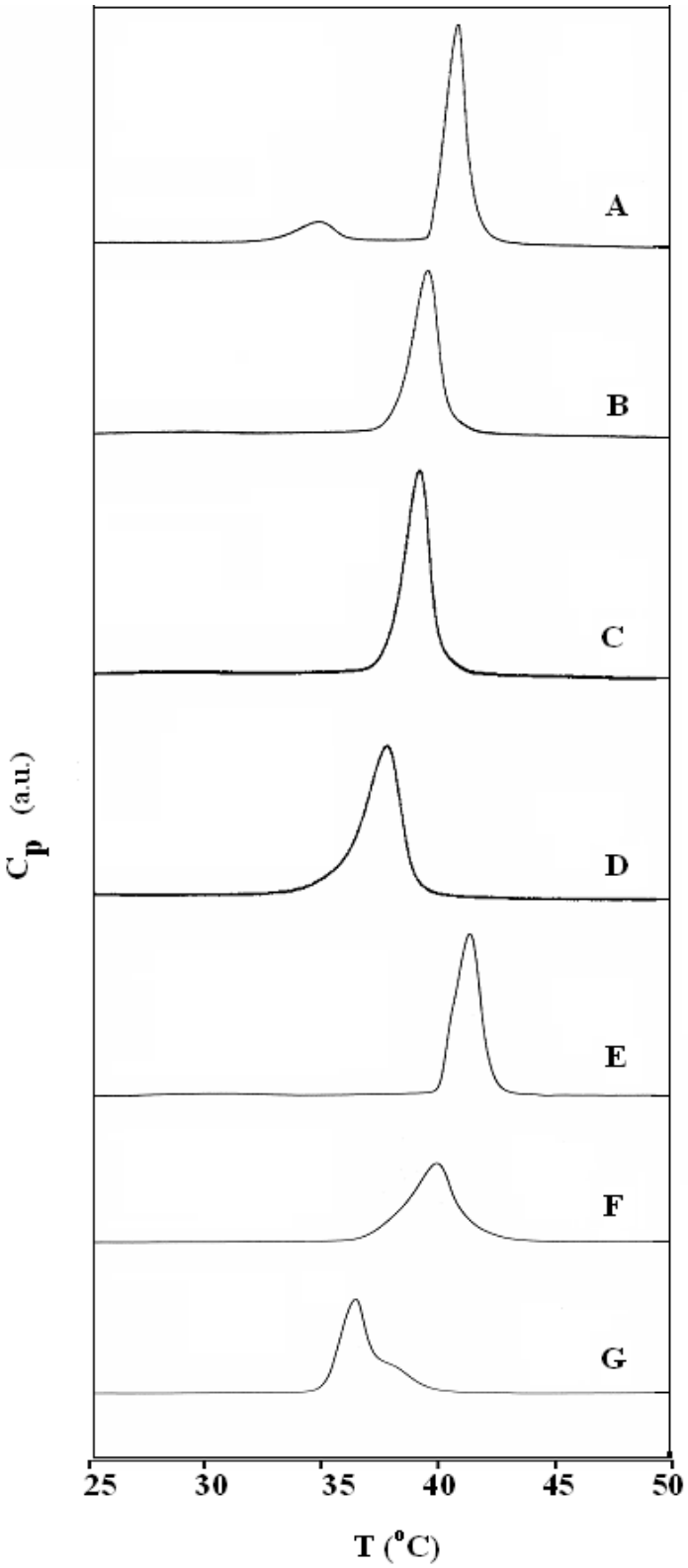

Figure 8. Normalized DSC scans of preparations containing (A) fully hydrated DPPC; (B) with χ=0.01 incorporated valsartan; (C) with χ=0.05 incorporated valsartan; (D) with χ=0.20 incorporated valsartan and (E) with χ=0.01 incorporated MMK3; (F) with χ=0.05 incorporated MMK3; (G) with χ=0.20 incorporated MMK3.

In the gel phase DPPC acyl chains tilt with respect to the bilayer normal in order to pack more tightly. In the ripple phase, headgroup acyl chain mismatch is accommodated by vertical displacement of the headgroups with respect to one another. Phospholipid bilayers of dipalmitoylethanolamine with smaller headgroups and no headgroup/chain mismatch, show no chain tilt, no ripple phase and no pretransition [33-36].

The incorporation of either drug perturbs the pretransition by affecting the chain tilt. This effect is concentration dependent. Thus, at x=0.01 they abolish the chain mismatch and therefore the pretransition and also cause a broadening of the main phase transition.

The transition temperature of the main phase transition is lowered in thermal scans containing valsartan or MMK3. Increase of the molar ratio x=0.05 causes a progressive increase in the breadth of the main transition and decrease of T_m. This is even more pronounced when x molar ratio is further increased (x=0.20). In the case of lipid bilayers containing valsartan a shoulder on the right side of the main phase transition peak is observed indicating phase separation in the membrane bilayer. Comparative diagrams of T_m , $T_{m1/2}$ and ΔH vs. the molar ratio x show that valsartan exerts more pronounced effects on the broadening of the phase transition and lowering of the main phase transition as well as on the increase of the enthalpy change (Figure 9). Valsartan thermal scans resemble closely those published for losartan using identical concentrations [27].

Increase of ΔH values for additives and other substances incorporated in lipid bilayers have been already reported in the literature and interpreted as an induction of partial or total interdigitation.

Raman Spectroscopy: Raman spectroscopy is suitable to reveal the *trans-gauche* transformations of the alkyl chain in the presence and absence of additive relative to the temperature changes. The vibration modifications of the different segments of lipid bilayers when the drug is incorporated provide information on the topography and on the efficiency of perturbation of the drug molecule in the lipid bilayers.

Raman spectra of DPPC bilayers alone and in the presence of valsartan or MMK3 are obtained at a temperature range of 27-50 ^{o}C. Representative spectra of DPPC alone and those containing MMK3 or valsartan at x=0.05 and x=0.20 above phase transition temperature (43 ^{o}C) are shown in Figure 10.

The methylene C-H stretching-mode region 2800-3100 cm^{-1} provides the most intense bands in the Raman spectrum of lipid samples and has been commonly used to monitor changes in the *lateral-packing* properties and *mobility* of the lipid chain in both gel and fluid crystalline unilamellar and multi-lamellar bilayer systems. In particular, the 2850/2880 peak height intensity ratio reflects primarily *inter-chain* interactions whereas the 2935/2880

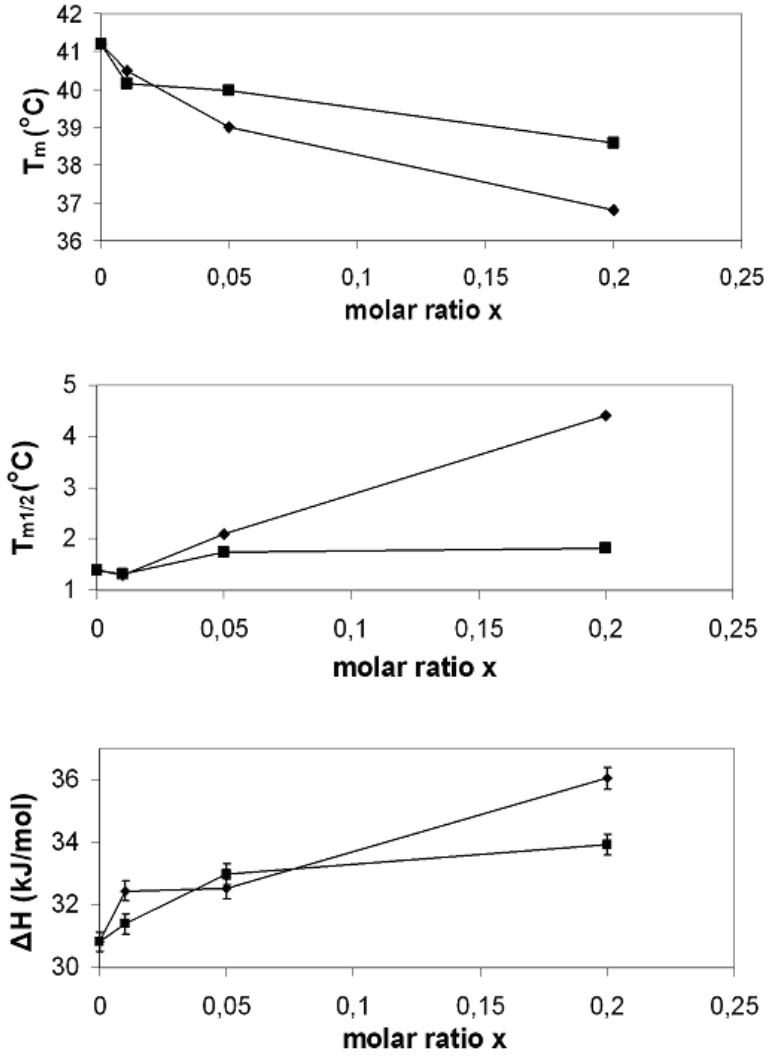

Figure 9. Comparison of the DSC variables T_m, $T_{m1/2}$ and ΔH vs. molar ratio between unloaded DPPC/ water bilayers and DPPC/ water bilayers containing valsartan or MMK3 The first points in the left (at zero molar ratios) represent measurements in unloaded DPPC/ water bilayers.

intensity ratio indicates effects originating from changes in *intra-chain gauche*/*trans isomerisation*. Thus, although the C-H stretching mode region consists of many superimposed vibrational transitions, the peak height intensity ratios described above provide a sensitive probe combining intramolecular (conformational) chain disorder together with intermolecular chain-chain packing disorder.

Figure 11(left) shows changes in 2935/2880 peak height intensity ratio caused by valsartan and MMK3, when they are incorporated in DPPC bilayers. The results show that valsartan and MMK3 cause a decrease in the gauche/trans ratio especially in the fluid phase, with valsartan to exert a more pronounced effect. Similar effects in the same phospholipid bilayers were

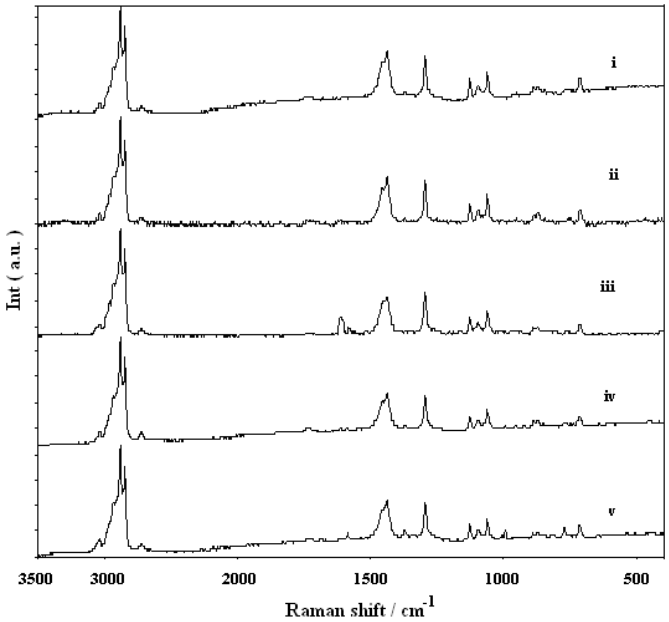

Figure 10. Raman spectra of (i) fully DPPC bilayers; (ii) containing x=0.05 incorporated valsartan; (iii) containing x=0.20 incorporated valsartan; (iv) containing x=0.05 incorporated MMK3; (v) containing x=0.20 molar ratio of MMK3 at 27 ^{o}C.

also observed for glycerol and interpreted as interdigitation effect [1]. The obtained results are also in accordance with DSC data which clearly show that the two drugs cause an increase in the ΔH attributed to the induction of partial or total interdigitation.

The C-C stretching mode region in the 1050-1150 $\tilde{cm}^{-1}$ spectral region directly reflects *intramolecular trans-gauche* conformational changes within the hydrocarbon chain region of the lipid matrix.

Figure 11 (right) shows the changes in $I_{1090/1130}$ intensity ratio caused by valsartan or MMK3 when they are incorporated in DPPC bilayers. The results show again that valsartan broadens more the phase transition than MMK3 and lowers its value in accordance with DSC data. In addition, induces lower *gauche/trans* ratio probably due to the interdigitation of the alkyl chains of the phospholipids. It appears that this ratio is more sensitive than $I_{2935/2880}$ to detect changes in *gauche/trans* ratio due to the presence of a drug.

Figure 12 shows the lineshape modifications and wavenumber changes of the peak at 714 cm^{-1} corresponding to C-N stretch vibration observed in DPPC bilayers at a temperature of 43 ^{o}C. The peak shifts to higher values when valsartan or MMK3 is incorporated in the lipid bilayers. This is indicative that both molecules interact with head-group. For comparison we tested the effect of losartan at the same peak (data not shown). Losartan caused the most significant shift. This is attributed to the fact that is

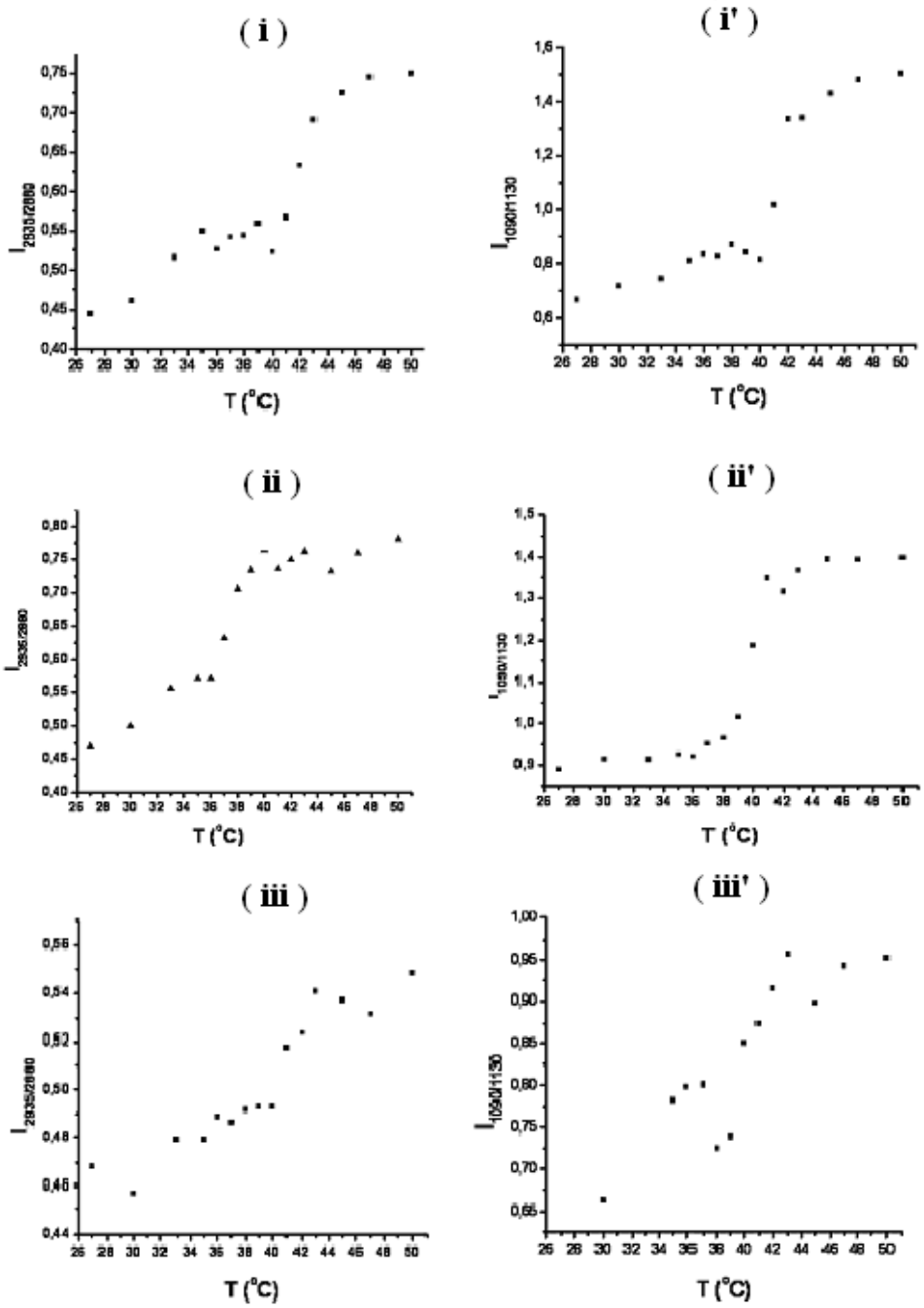

Figure 11. (left) $I_{2935/2880}$ and (right)$I_{1090/1130}$ vs temperature graphs for (i) fully hydrated DPPC bilayers (squares); (ii) DPPC bilayers containing x=0.2 valsartan (solid balls) and (iii) DPPC bilayers containing x=0.2 MMK3 (diamonds).

administered as a salt. The acidic group of losartan, tetrazole, is in an anionic form and therefore electrostatically interacts with the positive charge of the zwitterionic polar head-group of dipalmitoylphosphatidylcholine. This observation supports previously studies of losartan in the same bilayers using molecular modeling studies and solid state NMR spectroscopy [26].

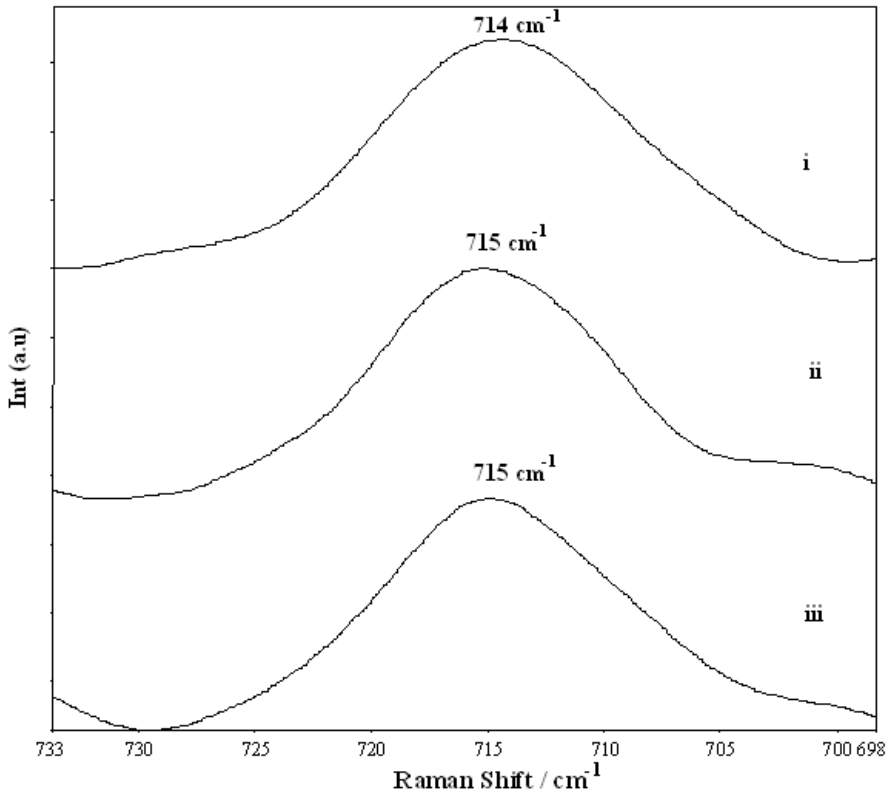

Figure 12. Lineshape modifications and wavenumber changes of the absorbed peak at 714 cm^{-1} corresponding to C-N stretching vibration observed at a temperature of 27 ^{o}C; in fully hydrated DPPC bilayers (top) added x=0.20 MMK3 (middle) and added x=0.20 valsartan (bottom).

^{13}C high resolution NMR spectroscopy

^{13}C MAS/NMR spectroscopy was applied to study the interactions of losartan with membrane bilayers. When a drug molecule is incorporated into the membrane bilayer, one can observe: (a) changes in peak intensity and line-width due to modified membrane fluidity; (b) changes in chemical shift values of individual carbon nuclei for the membrane lipid due to modified phase transition profiles; and (c) appearance of a specific subset of peaks from the carbon nuclei of the incorporated drug molecule.

Solid-state ^{13}C T_1 experiments were run at gel (25 $^{\circ}$C), and at liquid crystalline phase temperatures (43 $^{\circ}$C) of the DPPC bilayers alone and with 20 mol% losartan in an attempt to examine quantitatively the dynamic effects caused by losartan into the membrane bilayers (Fig. 14). Generally, an increase of T1 values both in preparations of lipid bilayers without and with the presence of drug molecule is observed as the temperature increases. At 25 and 43 $^{\circ}$C the incorporation of the drug does not cause significant changes in the T1 values of the resonances corresponding to the head-group (54.6, 60.1, 66.6 ppm) and

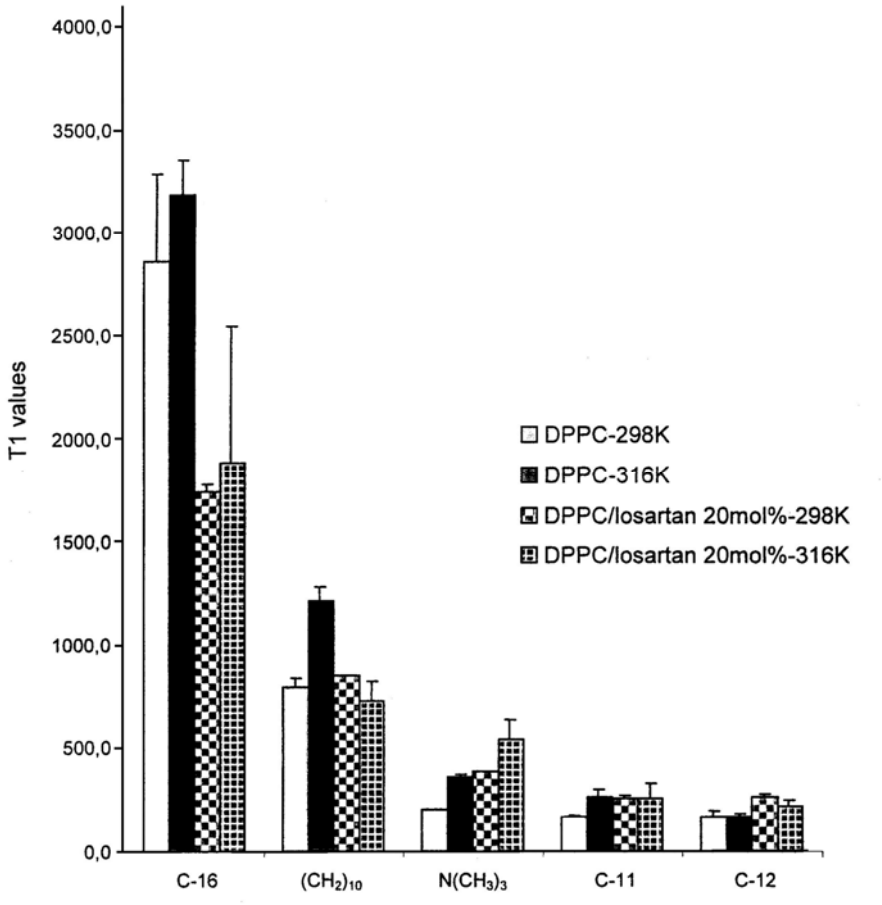

Figure 13. T_1 values for representative observed resonances of DPPC bilayers without and with 5 mol% and 20 mol% concentration of losartan at 298 K and 316 K.

glycerol backbone of DPPC bilayers. Concerning the major hydrophobic core (31.2 ppm) the insertion of the drug at gel phase does not affect significantly the T_1 values of the peak corresponding to alkyl chains of DPPC bilayers while in the liquid crystalline phase it causes significant decrease. In addition, the presence of the drug induces a significant decrease of the T_1 values of the resonance (14.3 ppm) corresponding to the terminal methyl group. The NMR results are in agreement with those of ESR reported by Theodoropoulou and Marsh and show that the presence of the drug causes a decrease of chain mobility of phosphatidylcholine bilayers both in gel and liquid phases of membrane bilayers [27].

^{31}P Solid State Static NMR and X-ray spectroscopy's

^{31}P is used extensively to study the polymorphic states of lipid bilayers. Recently, a methodology based on ^{31}P NMR CP (cross polarization) broadline simulations was developed in our laboratory [37] aiming to acquire

important information on drug:lipid bilayers interactions. Specifically, this methodology is of paramount importance if the drug molecules exert their action at the head group.

Few of the parameters developed in this methodology which may be associated with the induction of interdigitation are: *increase of the inhomogeneous broadening*, an indication that the molecule disturbs the conformation of the head group of the phospholipids; *increase of the homogeneous broadening* resulting in the decrease of the mobility of lipid bilayers. *Increase of the collective tilt* is asscociated with higher order in the DPPC/water bilayer system. We are in progress to study the effects of AT_1 antagonists using the above methodology in order to confirm our data based on other methodologies. In addition, in collaboration with Institute of Biophysical Research of Graz, Austria we have initiated X-ray diffraction studies to study the possible interdigitation effect of AT_1 antagonists by observing the d-spacing decrease.

In conclusion, all the applied methodologies point out that AT1 antagonists induce interdigitation in lipid bilayers. There is a lot of research work to be carried out in the future. First more AT_1 antagonists must be tried to establish the already obtained results. The effects of AT_1 antagonists in a variety of phospholipids alone or mixtures should be studied with the presence and the absence of cholesterol to investigate if the effect is lipid dependent and also examine the role of cholesterol.

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