Differential scanning calorimetry investigation of the interaction of cationic amphiphilic model compounds and muscarinic allosteric agents with phospholipid bilayers

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathemathisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn 2004

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der
Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der Promotion: 29. Juli 2004

Die vorliegende Arbeit wurde in der Zeit von Juli 2000 bis März 2004 in der Pharmakologie und Toxikologie des Pharmazeutischen Instituts der Rheinischen Friedrich-Wilhelms-Universität zu Bonn unter der Leitung von Herrn Professor Dr. med. Klaus Mohr angefertigt.

Danksagung

Mein besonderer Dank gilt Herrn Prof. Dr. Klaus Mohr für die hervorragende Betreuung meiner Arbeit. Seine Arbeitsweise, seine Geduld sowie seine Diskussionsbereitschaft werden mir immer als Vorbild gelten.

Herrn Prof. Dr. Klaus Jürgen Steffens und Herrn Prof. Dr. Heinz Bönisch danke ich für die Übernahme des Korreferates.

Herrn PD. Dr. Hubert Rein danke ich für die Einarbeitung in die Methodik der Differential Registrierende Kalorimetrie und seine ständige Diskussionsbereitschaft.

Ebenso dankbar bin ich Herrn Dr. Christian Klein für seine freundschaftliche Unterstützung and Herrn Dr. Andreas Dittmann für seine Hilfsbereitschaft bei computertechnischen Herausforderungen.

Allen derzeitigen und ehemaligen Mitarbeitern der Abteilung Pharmakologie und Toxikologie danke ich für die angenehme Arbeitsatmosphäre.

Für die finanzielle Förderung meiner Arbeit danke ich dem Katholischer Akademischer Ausländer Dienst. Insbesondere möchte ich mich hier bei Frau Simone Saure, Herrn Dr. Thomas Scheidtweiler und Herrn Dr. Hermann Weber für ihre Gesprächs- und Hilfsbereitschaft bedanken.

1. Introduction

1.1 Biomembranes

Most drug substances elicit their therapeutic activity through a direct specific interaction with receptor-proteins. The majority of these receptors are membrane bound. These receptors usually have hydrophilic and hydrophobic domains that help in anchoring them in cell membranes in addition to their ligand binding sites. Parts of the hydrophobic domains are usually stabilised through van der Waals interactions with the hydrophobic components of the phospholipids in biomembranes, whilst the parts situated in the interior of the cell are stabilised by the cytoskeleton. The receptors receive and amplify regulatory signals. However, receptor-proteins are by no means the only site of action of drug substances; many drugs do act on other cellular structures. Moreover, the action of receptor proteins is not only influenced by other cellular structures, but is dependent on the presence of these latter for signal transduction. Through signal transduction pathways, numerous essential functions are controlled in all tissues and the structures involved for this transduction are ubiquitous throughout the animal kingdom.

Apart from representing a physical and chemical barrier and being the carriers of surface receptors, biomembranes are extremely dynamic structures that are intricately connected with the cell metabolism (Gross et al., 1989). The cell membrane forms a selectively permeable barrier and is also a carrier of enzymes. It builds the morphological and functional basis for excitability (Gross et al., 1989; Lodish et al., 2003) and it provides an anchor for carriers of biochemical characteristics, such as the major histocompatibility complex, that are responsible for immunological individuality (Köhler and Eichmann, 1988). The barrier function ensures the parallel and simultaneous, and yet separate running of the various cellular metabolic activities in the different organelles.

1.1.1 Composition of bio-membranes and molecular structure of phospholipids

The principal components of biomembranes are phospholipids, proteins and to a smaller extent sterols and carbohydrates (Leistner and Breckle, 1992; Lodish et al., 2003). The individual proportions of these latter can vary considerably. They are cell-, organelle- and organism-specific and their composition depends on membrane

function. While the lipids and proteins are present in the membrane as individual constituents, the carbohydrates are covalently bonded to the lipids (glycolipids) or to the proteins (glycoproteins). The proteins can also be characterised based on their arrangement in the cell membrane, a distinction being made between integral and peripheral membrane proteins. Peripheral proteins are bound on the membrane surface while integral proteins penetrate deeper into the membranes (Gross et al., 1989; Leistner and Breckle, 1992). These latter may span the membrane. The peripheral proteins generally do not interact with the hydrophobic core of the bilayer. The integral proteins on the other hand do interact and are stabilised through van der Waals interactions with the non-polar lipid domains of the phospholipids.

Phospholipids are amphiphilic molecules comprising a phosphate group or phosphate ester that is further esterified with a diacylglycerol. The two further hydroxyl groups of the glycerol are esterified with long chain monocarboxylic acids. The ester component bound to the phosphate group is usually a hydrophilic compound. Among the most important of these in living organisms are ethanolamine, serine, choline and the sugar derivative inositol. The hydrophilic molecule component on the one hand and the fatty acid hydrocarbon chains on the other hand are responsible for the amphiphilic character of the phospholipid.

The membrane composition is influenced by external factors such as temperature, dietary composition and pH value of the surrounding medium. The composition is modified to adapt to fluctuations in external milieu, chiefly through changes in lipid content of the membrane. Among the most important lipids in animal cell membranes are phosphatidylcholine (lecithin), phosphatidylethanolamine (cephalin), phosphatidyl-serine and phosphatidylinositol. The structures of a phospholipid, and a space-filling model of a typical phospholipid bilayer are shown in the next two figures.

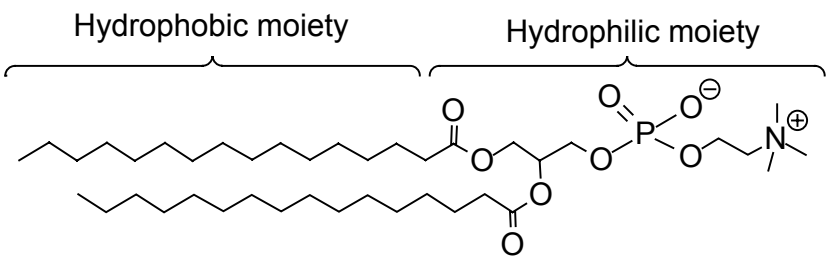


Figure 1-1 Structural formula of the phospholipid dipalmitoylphosphatidylcholine. As explained in the text above, the compound consists of a hydrophobic and a hydrophilic part.

Figure 1-2 illustrates the manner in which phospholipids are arranged in a bilayer.

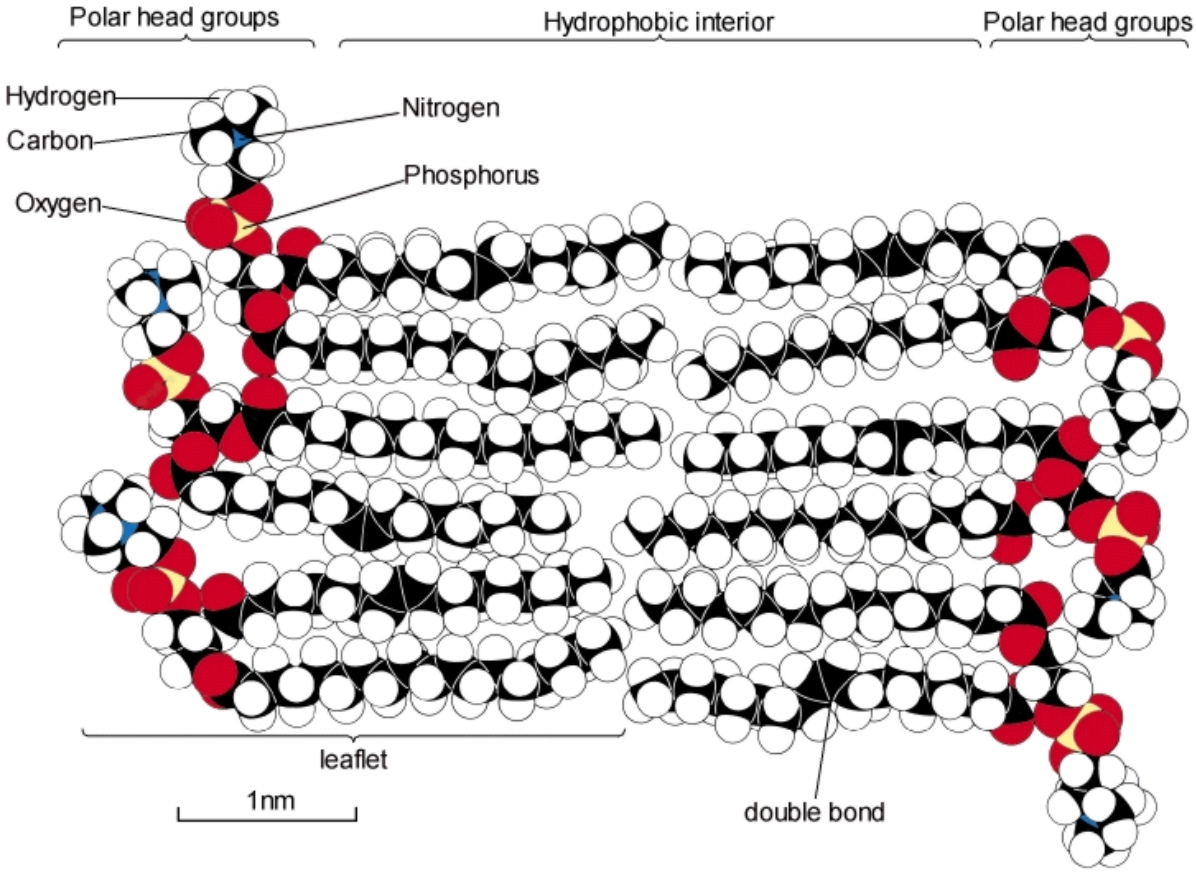


Figure 1-2: A space-filling model of a typical phospholipid bilayer. The fatty acyl side chains generate the hydrophobic interior. Some of these chains have double bonds. The different polar head groups all lie on the outer, aqueous surface of the bilayer (adapted from *Biochemistry,* 4th ed., 1995, W.H Freeman and Company).

In phospholipid bilayers, thermal motion permits the individual phospholipids to diffuse laterally within the membrane leaflet, with the fatty acyl chains remaining in the hydrophobic interior of the membranes. The membrane is best envisioned as, and represented using the fluid mosaic model postulated by Singer and Nicolson in 1971. According to this model, the membrane is viewed as a two-dimensional mosaic of laterally mobile phospholipid and protein molecules.

1.1.2 Importance of membrane interactions

Phospholipids play a vital role in numerous cellular activities. From genetic analysis, dynamin, a guanosine triphosphatase (GTPase) is thought to play a role in endocytosis. Previous studies have stressed an in vitro association with microtubules, and additional evidence suggests that dynamin associates with membranous organelles. In an analysis of the enzymatic and membrane binding properties of dynamin carried out by Tuma et al. (1993) it has been found that the acidic

phospholipids phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol are able to stimulate GTP hydrolysis in a manner similar to activation previously shown with microtubules. These results suggest that phospholipid membrane components could be responsible for some aspects of the regulation of dynamin function in vivo (Tuma et al., 1993; Achiriloaie et al., 1999).

Also, receptor-mediated endocytosis via clathrin-coated vesicles has been extensively studied and it has been found that some components of the endocytotic machinery interact with inositol polyphosphates and inositol lipids in vitro, implying a role for phosphatidylinositols in vivo. A specific adaptor protein complex binds phosphatidlinositol-4,5-bisphosphate among others. Using high affinity and high specificity probes in combination with a perforated cell assay Jost et al. (1998) provided direct evidence that phosphatidylinositol-4,5-bisphosphate is required for both early and late events in endocytotic coated vesicle formation.

Electrostatic binding of proteins and basic polypeptides to the (anionic) polar groups of phospholipids shifts the temperature of the gel-liquid crystal transition (section 1.2) and also affects the energy barrier for reorientation (Chapman and Urbina, 1974). This behaviour may be relevant to the interpretation of thermal transitions observed with some biomembranes.

A large number of pharmaceutically active compounds have a high affinity to acidic phospholipids; good examples are the cationic compounds lidocaine, propranolol, and gentamicin. These drugs influenced the lipid dynamics of liposomes composed of phosphatidylcholine and the acidic phosphatidylglycerol (Jutila et al., 1998).

The importance of drug-membrane interactions cannot be overemphasised. The cytotoxic and cytolytic effect of many bacterial and serpent toxins, including the use of bacterial toxins as specific tools in biochemistry and experimental pharmacology stresses the importance of biomembranes as targets for xenobiotics.

The far-reaching pharmacological and toxicological implications of interactions with membrane components have led to many experimental techniques being developed to investigate these interactions.

1.2 Phase transition and liposomes

By virtue of their constitution, phospholipids spontaneously form symmetric bilayers in aqueous solution. These are normally two molecules thick and have their fatty acid hydrocarbon side chains facing each other, forming an interface with a hydrophobic core. The hydrophilic polar head groups pack together facing the aqueous solution.

This is illustrated in figure 1-2 in the previous section. In sufficiently high concentrations, the phospholipids spontaneously seal to form closed structures, separating two aqueous compartments. Forming sheets with open ends in contact with the aqueous solution would lead to instability; spherical structures with no ends on the contrary reduce the potential energy of the system and thus stabilises the bilayer structure. The propensity of phospholipids to form these structures has been used as an important experimental and clinical tool.

One consequence of the packing of the fatty acyl chains within the centre of a phospholipid bilayer is an abrupt change in the latter's physical properties usually over a very narrow temperature range. At low temperatures, the phospholipid bilayer possesses a gel-like consistence with the chain regions in a highly ordered crystalline state. Here, the hydrocarbon chains are predominantly in the trans conformation and are stabilised by van der Waals forces of attraction. On heating, it experiences a phase change at a specific temperature, melting to become more fluid with the phospholipid molecules exercising increased mobility. This phase transition is accompanied by a change in conformation of the hydrocarbon chains. Through the uptake of thermal energy, a considerable amount of gauche-conformers and other energetically unfavourable conformations are formed. The energy uptake for this process can be measured. This phase transition can be observed in a suspension of liposomes (see below) composed of phospholipids, when heated. The "melting temperature" (or phase transition temperature) at which this transition occurs is characteristic for each phospholipid.

In view of the importance of interactions with biomembranes in living organisms, in-vitro preparations of phospholipid bilayers often serve as models for the investigation of membrane properties and the interactions of biomembranes with drug substances. The melting process can be used in investigating interactions of drug substances with phospholipid bilayers, since the presence of the former influence the phase transition temperature. The effect of the substance varies from one phospholipid to another. It has been postulated that through interaction of drug substances with the lipid bilayers, domains are formed containing solely phospholipid molecules and others containing both test substance and phospholipid molecules in varying molar ratios.

For the experimental purpose, liposomes are normally used. These are vesicles composed of one or more concentric phospholipid bilayers, with the latter alternating with an aqueous layer. In addition to their experimental importance, the use of liposomes as therapeutic tools, especially as carriers of cytotoxic drugs in drug

targeting is also increasing (Schreier, 1982; Boggs et al., 1987; Yamazaki et al., 2000; Oku et al., 2001; Matsuura et al., 2003; Immordino et al., 2003; Cattel et al., 2003).

1.2.1 Biomembranes or artificial phospholipid membranes

The question arises if it would be more advantageous to use biomembrane extracts or artificial phospholipid membranes for investigative purposes. Formerly, isolated biomembrane extracts were used in investigations involving phospholipid membranes. Preparations from erythrocytes were most often used; not only do they contain most of the components present in intact biomembranes, they are also easily extracted from whole cells. However, results from the investigation of interactions between various test substances and phospholipids would be affected by the presence of other membrane components. It would not be possible to find out the pharmacological effects brought about solely by interactions with phospholipids. It was thus necessary to device an experimental model with pure phospholipids specifically for the investigation of the phospholipid-substance interaction and so far this can best be done using artificial phospholipid membranes. This is without doubt a simplified model compared with the prevailing conditions in actual biomembranes with regards to composition, but the model has the advantage in that it simplifies the interpretation of the observed results.

While earlier experiments were conducted using membrane models with the prior purpose of investigating the relationship between structure and lipophilicity of the test substances with phospholipids, the objectives of these experiments have been changing in recent years. There are indications presently that membrane phospholipids may play a more active role in activities that take place involving other membrane components. As described above, there is evidence that phosphatidylinositol-4,5-bisphophate is required for both early and late events in endocytotic coated vesicle formation (Jost et al., 1998). The phospholipids may not necessarily be direct targets of drug action, but they do seem to be relevant for interactions leading to various cellular activities.

Numerous experiments have been carried out that investigate the interaction of various substances with phospholipid membranes by various research groups (Ladbrooke et al., 1968; Hauser et al., 1969; Phillips et al., 1970; Cater et al., 1974; Lee, 1975b; Frenzel et al., 1978; Surewicz and Leyko, 1981; Kursch et al., 1983; Hanpft and Mohr, 1985; Girke et al., 1989; Borchardt et al., 1991; Mohr and Struve, 1991; Klein et al., 2001).

1.3 Allosteric modulators

Research has been carried out on allosteric modulation of subtype 2 muscarinic (M_2) acetylcholine receptors using binding studies by members of our research group among others. In theory, the therapeutic use of allosteric modulators should bring about a number of advantages due to a ceiling level to the allosteric effect, and the ability to enhance the binding of the endogenous acetylcholine, leading to the potential for greater receptor selectivity (Christopoulos and Kenakin, 2002). This would mean less side effects and better therapy control. There exists great structural variation among the substances found to modulate the binding of orthosteric ligands allosterically. The allosteric behaviour of these compounds also varies. One such modulator investigated is the bisammonium compound W84 (section 2.3.2.1). Results from equilibrium binding experiments showed that the substance reduces the binding of the prototype muscarinic receptor antagonist, N-methyl scopolamine (NMS) on M_2 receptors. It inhibits the association of the radio-labelled antagonist [^{3}H]NMS to M_2 Receptors more than it retards the dissociation of the antagonist from its M_2 binding (Jepsen et al., 1988; Tränkle et al., 1996).

Experiments were also performed with derivatives of this compound. A silicon-containing derivative, TD5, had a completely different behaviour compared to that of the parent compound W84 (Duda-Johner, 2002). The substance naphmethonium (section 2.3.3.1), a further derivative in which a phthalimidopropyl residue was replaced through a bigger naphthylimido-ß-dimethylpropyl residue, was also tested to permit a direct comparison with the prototype modulator W84 with regard to the relationship between size and lipophilicity against the induced effect through interaction with the phospholipid bilayer.

1.4 Goals and objectives

One of the major objectives of this work was using these W84-derivatives in this phospholipid interphase model to investigate if a relationship could be established between the interphase activity and the allosteric action.

Based on the heterogeneity of the structure of allosteric modulators, it is easy to postulate that it is not exclusively the binding onto a specific site that brings about their effect. It is conceivable that unspecific interaction with other parts of the receptor protein and/or the surrounding phospholipid membrane, particularly negatively charged membrane components could be involved. This should not be surprising, considering that it has been shown that acidic phospholipids are able to stimulate GTP-hydrolysis (Tuma et al., 1993). Small molecules may react only with specific domains in the receptors but parts of larger, longer and more stretched-out molecules may exercise an effect on sites other than the classical ligand-binding site. The $ß_2$-receptor agonist salmeterol is supposed to exercise its effects through binding at the agonist binding domain as well as interacting using its side chain with other receptor components (Christopoulos and Kenakin, 2002).

The characteristics of interaction with orthosteric receptor ligands revealed differences among the modulators, leading to the terms “typical” and “atypical” being applied for the classification of the various modulators (Traenkle et al., 2003).

Typical allosteric modulators are ligands that bind normally at the common allosteric site. Antagonists binding to this very site can displace these modulators (Ellis and Seidenberg, 1992). While W84 and Wduo3 (section 2.3) could be identified as ligands at the common allosteric site in binding studies, this was not the case with the substance Duo3 (Dittmann, 2003).

This work was divided into two parts: firstly, we aimed at establishing a relationship between depth of penetration of cationic amphiphilic model substances into bilayers and their influence on the phase transition temperature, T_t. The penetration depth would invariably depend on a number of factors including the length of the molecule, its lipophilicity, its structure and charge and also the charge density. With the aid of substances with a simple molecular structure, not only could a relationship be easily established but also a number of the factors influencing the depth of penetration could be kept constant. Beginning with a simple phenylpropylamine, related substances where synthesised through a continuous and systematic introduction of varied

substituents in different positions of the parent compound by D. Heber and M. Klingmüller (Pharmaceutical Chemistry, University of Kiel). These substances, some of which had previously been investigated, were systematically studied with respect to their effects on phospholipid phase transition behaviour.

Based on these findings, we then aimed at conversely determining the degree of interaction and depth of penetration of the mentioned typical and atypical modulators of muscarinic acetylcholine receptors with phospholipids.

Beginning with the typical M_2 allosteric modulator, W84, and going over to the atypical silicon-containing derivative, TD5, and further allosteric modulators on M_2 receptors, an attempt is made to investigate these modulators using the interface model.

2. Materials and Methods

2.1 Materials

The used phospholipids were obtained from Sigma-Aldrich Chemie, Schnelldorf, Germany.

2.1.1 Phospholipids

Those used in the experiments were dipalmitoyl L-α-phosphatidylcholine (1,2 – dihexadecanoyl-sn-glycero-3-phosphocholine [DPPC], synthetic and approximately 99% pure) and the sodium salt of 1,2 - dihexadecanoyl-sn-glycero-3-phosphate (dipalmitoyl L-α-phosphatidic acid, DPPA, synthetic and approximately 99% pure).

2.1.2 Further reagents

L-Histidine (> 99% pure), TES (N-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (> 99% pure) and aqua pro analysi were obtained from Merck KgaA, Darmstadt, Germany.

2.1.3 Buffer solution

The buffer solution used was a 14mM TES-histidine buffer with a pH value of 6.

2.2 Data analysis

The analysis of the acquired data and their eventual representation as thermograms was carried out using the program Pyris® Software for Windows®, version 3.72, Perkin Elmer, USA.

Statistical evaluation was performed using Graph Pad Instat, version 3.05 and the dose-effect curves were plotted using Graph Pad Prism, version 3.00 (Graph Pad Software, San Diego, USA),

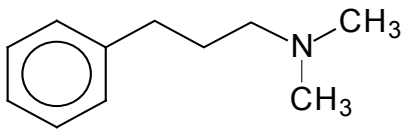
Chemical structural formulae were constructed using ChemWindow®6.0 (Bio-Rad Laboratories, Philadelphia, USA).

2.3 Test substances

2.3.1 Drug substances

Various research groups have previously calorimetrically investigated a range of drug substances. However, most of the equipment used so far differed from that being used in the experiments carried out in this work. It was therefore deemed necessary to use a substance previously investigated to validate the apparatus and the method. For this purpose, propranolol was used.

2.3.2 Phenylpropylamines



KH210

The structure of the parent compound KH210 is shown above. Derivatives of the compound and further investigated aromatic propylamines are shown in the appendix. These substances were used as their hydrochloride salts and were readily soluble in buffer solution. The KH compounds were originally synthesised by Dr. Klingmüller and Prof. Heber of the Pharmaceutical Institute, University of Kiel. Subsequent synthesis was carried out by Christian Klein, formally a member of the research group of Prof. Dr. Ulrike Holzgrabe, Pharmaceutical Chemistry, University of Würzburg, and now of the Eidgenoessische Technische Hochschule, ETH, Zürich-Switzerland.

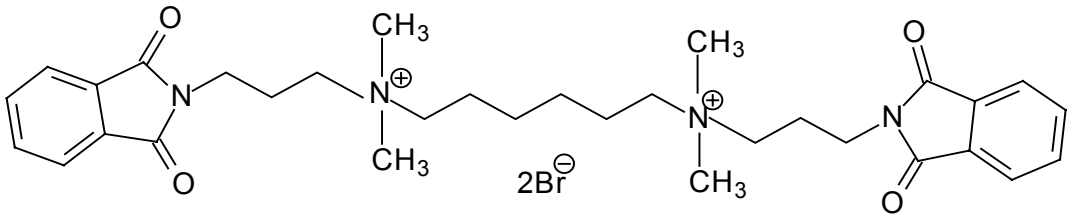
2.3.3 Allosteric modulators

The allosteric modulators of muscarinic acetylcholine receptors investigated in this work as well as their structural formulae are given below.

2.3.3.1 Alkane-bisammonium-type modulators

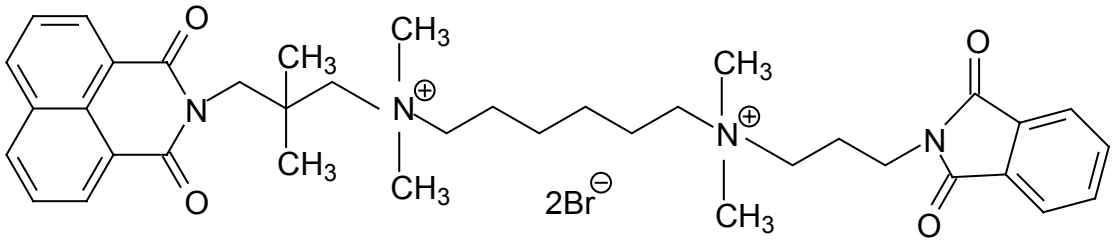
W84 (Hexane-1,6-bis(dimethyl-3´-phthalimidopropyl-ammonium dibromide))

The substance W84, an archetypal muscarinic acetylcholine receptor modulator, was synthesised and donated by Dr. J. Pfeffer (Institute of Pharmacology, University of Kiel), using the method established by Wassermann of the Institute of Pharmacology, University of Kiel, in 1970.



W84

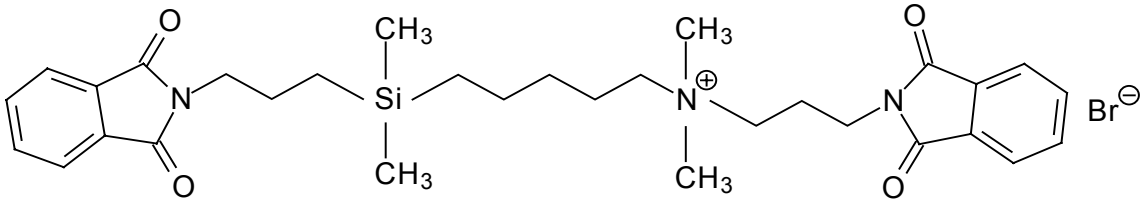
Naphmethonium



Naphmethonium

Tests were also carried out with the W84 derivative naphmethonium (MM3A). The difference in the structures of this compound compared with W84 is evident. The substance was synthesised by Mathias Muth, a member of the research group of Prof. Dr. Ulrike Holzgrabe, Pharmaceutical Institute of the University of Würzburg.

2.3.3.2 Silicon-containing derivative



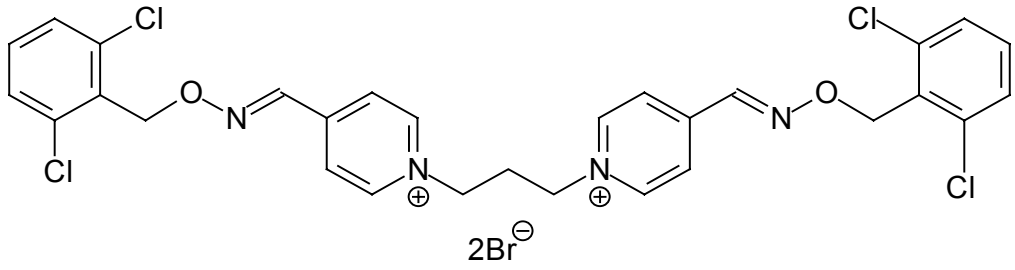
TD5

The compound TD5, a derivative of W84 was synthesised and donated by J. Daiss of the research group of Prof. Dr. R. Tacke, Institute of Inorganic Chemistry, University of Würzburg, Germany.

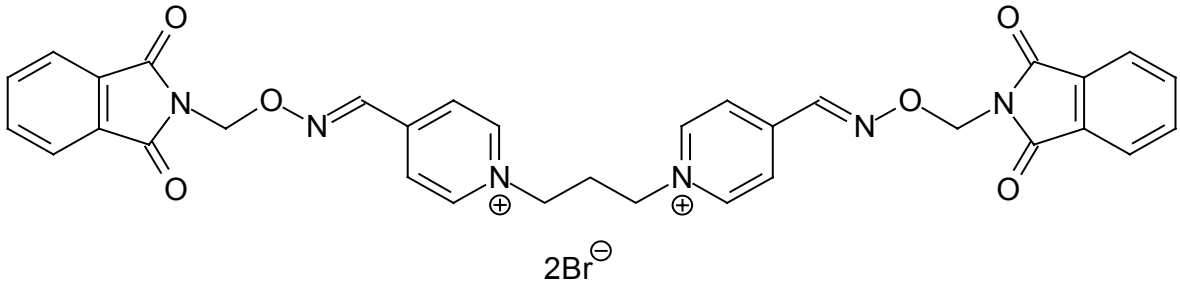
2.3.3.3 Bispyridinium-type modulators

The compounds Duo3 (1,1'-(1,3-propandiyl)-bis[4,4'-(2,6-dichlorbenzoxyl)-iminomethyl-pyridinium]-dibromide) and WDuo3 (1,1'-(1,3-propandiyl)-bis[4,4'-phthalimidomethoxyl-

iminomethyl-pyridinium]-dibromide), whose structures are shown below, were synthesised by members of the research group of Prof. Dr. Ulrike Holzgrabe, Pharmaceutical Institute of the University of Würzburg.



Duo3



WDuo3

2.4 Test principle and apparatus: Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry measures the heat flow associated with transitions in materials as a function of time and temperature. The technique provides qualitative and quantitative information about physical and chemical changes that involve endothermic or exothermic processes, in other words changes in heat capacity, with the possibility of investigating minimal amounts of sample. A small sample quantity ensures uniform temperature distribution within the sample. The latter can be encapsulated in an inert atmosphere to prevent oxidation.

Further advantages include fast analysis time, easy sample preparation, applicability to both liquids and solids, measurements over a wide temperature range and an excellent quantitative analytical capability.

There are two types of DSC systems in common use (Figs 2-1 and 2-2).

In heat-flux DSC, the sample and reference are connected by a low-resistance heat-flow path (a metal disc). The assembly is enclosed in a single furnace. Enthalpy or heat capacity changes in the sample cause a difference in its temperature relative to

the reference. The temperature difference is recorded and related to enthalpy changes in the sample.

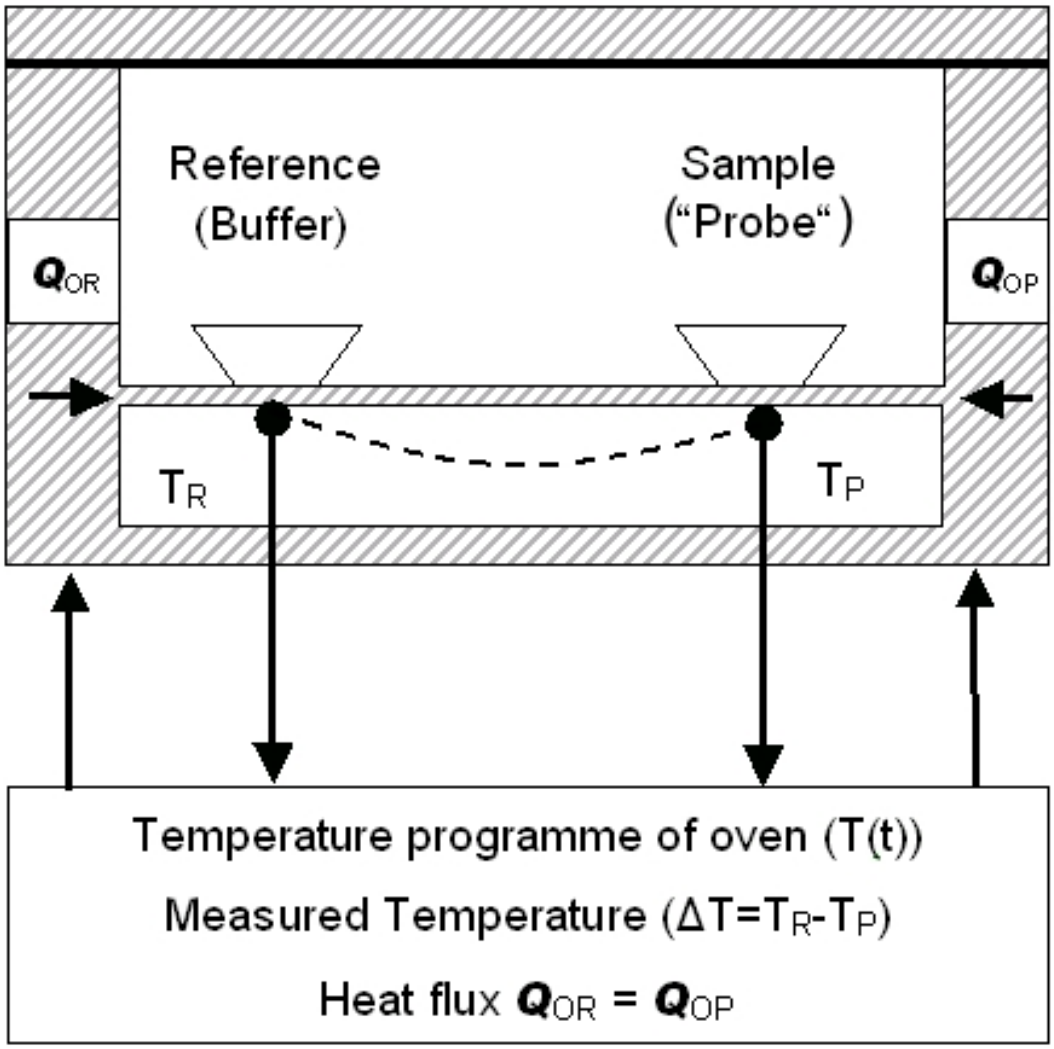


Figure 2-1: Schematic representation of heat-flux differential scanning calorimeter (not to scale). The arrows indicate direction and path of energy flow.
T_R = temperature of the reference, T_P = temperature of sample, $\dot{Q}_{OR}$ = heatflow to reference, $\dot{Q}_{OP}$ = heat flow to sample. The subscripted p is taken from the German and stands for "Probe", meaning sample in this context. Adapted from Ehrenstein et al. (1998).

In power-compensation DSC the temperatures of the sample and reference are controlled independently using separate, identical furnaces. The temperatures of the sample and reference are made identical by varying the power input to the two furnaces; the energy required to do this is a measure of the enthalpy or heat capacity change in the sample relative to the reference.

The energy difference required to maintain similar temperatures in both pans is recorded and registered in thermograms (Fig. 2-3). This difference is normally small, a

fact that is clearly demonstrated by the baseline. In the event of a transition, the difference increases and this is registered as a signal in the thermogram.

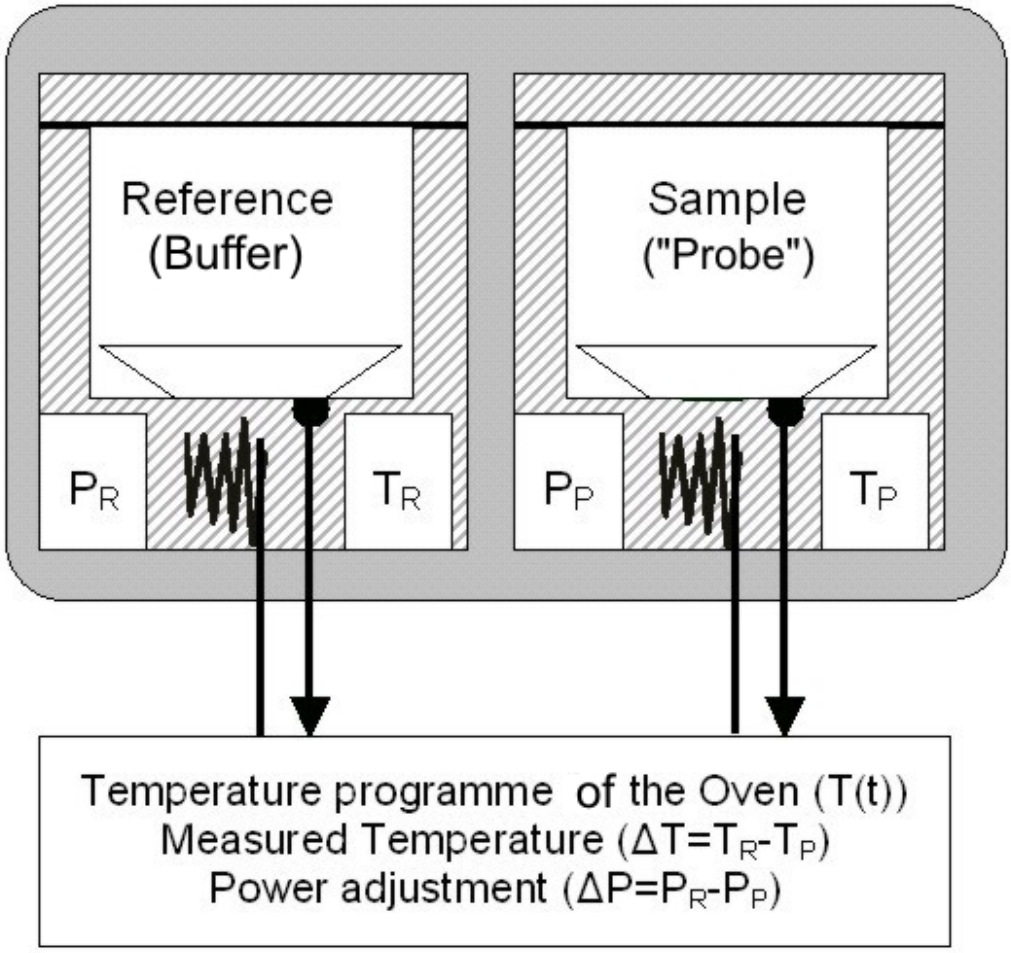


Figure 2-2: Schematic representation of power compensation differential scanning calorimeter (not to scale). T_R = temperature of the reference, T_P = sample temperature, P_R = power of reference oven, P_P = power of sample oven. The increased energy uptake of the sample during the phase transition can be compensated by increasing the power input through the heating coils situated beneath the pan holders.

2.4.1 Details on components of a thermogram

2.4.1.1 The baseline

In DSC, it is expedient to conduct experiments either isothermally or with the temperature changing at a constant rate. In the former case, the ordinate value would be plotted against time at isothermal temperature, whereas in the latter case it could be plotted against time or temperature. The latter case was applied in experiments carried out in this work. The baseline (Fig. 2-3) corresponds to the value recorded for

the calorimetric signal when no difference exists in the thermal power evolved or taken up by the pans, one of which contains the sample being investigated. The baseline can be visually estimated for sharp peaks without entailing large errors. For broad peaks it is difficult to qualitatively establish the baseline. Usually, a good approximation to the baseline is a straight line connecting the start and finish of the transformation. A straight line could conveniently link the baseline prior to and after the phase transition signals in most of the experiments carried out in this work. This made it easy in many cases to determine the onset temperature pretty accurately. With amorphous solids, there can be large differences in the vertical position of the baseline.

2.4.1.2 Onset and peak temperatures

The point of deviation from the baseline determines the onset temperature, the temperature on the abscissa scale resulting from the extrapolation of the intersection of the tangent drawn at the point of greatest slope on the leading edge of the peak with the extrapolated base line for the transition. In figure 2-3, these are denoted T_p and T_t for the onset temperatures of the pre- and the main transition peaks respectively. As the name implies, the peak temperature is that which corresponds to the highest point in the transition signal and is denoted T_{pm}. In the event that the curve is plotted with the endothermic heat flow recorded downwards, this occurs at the lowest point in the signal. The area under the transition signal determines the enthalpy change and is the area enclosed between the transition signal and the interpolated base line.

Figure 2-3 is a transition signal obtained from an original DSC measurement with DPPC showing typical peaks and illustrating the determination of the onset temperatures. As can be seen from the pre-transition peak, the deviation from the baseline is not always an abrupt one.

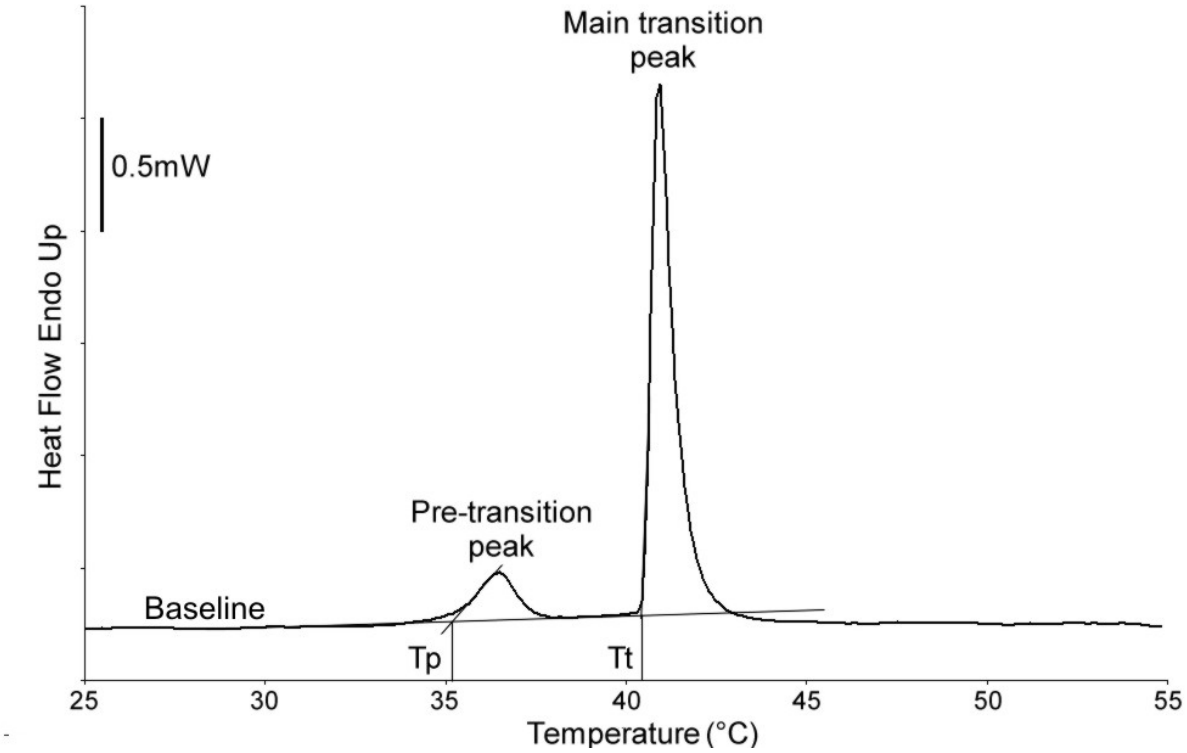


Figure 2-3: Original thermogram resulting from DSC measurement of pure DPPC liposomes. T_p and T_t denote the onset temperatures for the pre-transition and main transition respectively. ***Ordinate****:* endothermic heat flow. ***Abscissa****:* sample temperature in °C

2.4.1.3 Limit of detection

In calorimetry, the term “limit of detection” signifies the smallest heat quantity or thermal power that can be determined with reasonable certainty in a practical experiment conducted under specified conditions (Wadsö and Goldberg, 2001).

In practice, the whole signal is not described but only a definite temperature, referred to as the melting temperature, and if necessary the enthalpy change. The melting process begins at the onset temperature and continues until the total sample has melted at the peak temperature. The curve then descends to the baseline. Usually the extrapolated onset temperature T_t constitutes the “melting temperature”, although some groups use the peak maximum T_{pm}.

2.4.2 Temperature calibration

The device used was calibrated using substances with precisely known melting points. The calibration was done using the elements Mercury, Indium and Zinc. The melting points of these metals span the range from –39°C to 420°C, with Mercury having the

lowest melting temperature (–38.8°C) and Zinc the highest (419.6°C) among the used substances. Indium has the intermediate melting temperature of 156°C.

2.4.3 Thermodynamics background

The following are some equations used in characterising the quantities involved in the course of a DSC measurement. These selected equations were taken from “Praxis der Thermischen Analyse von Kunststoffen” by Ehrenstein et al., and from Wadsö and Goldberg’s IUPAC Technical Report, Standards in Isothermal Microcalorimetry (2001), (Ehrenstein et al., 1998; Wadsö and Goldberg, 2001).

The enthalpy, H, defines the sum of the internal energy and the product of the pressure and volume of a thermodynamic system, and it has the units of energy. At constant pressure and volume, the change in internal energy of a substance in the system solely defines the enthalpy change and this defines the energy output or uptake in a transition process. For practical purposes, the change in enthalpy, ΔH, is of greatest importance. It is given in Joules per gram.

$$\Delta H = \int c_p \cdot dT \qquad \text{Equation 1}$$

This is calculated from the deviation of heat flux from the baseline. The heat flux (Q) through the specimen is directly proportional to the latter’s specific heat capacity and is given by the following equation:

$$Q = m \cdot c_p \cdot v \qquad \text{Equation 2}$$

where m is the mass of the specimen, c_p is its specific heat capacity and v, the constant of proportionality is the heating rate. The specific heat capacity of a substance is the heat energy required to raise the temperature of a gramm of the substance by 1°C at constant pressure. Based on this equation, the relationship between the parameters, heating rate and sample mass is evident.

The rate of temperature change of the sample is constant along the baseline. This changes during the transition and is reflected in a change in Q. Using Q and the sample mass, ΔH can be calculated.

The plot displays heat flow per gram of material against temperature. The mass of the sample is known, the required energy is delivered by the DSC device through the microprocessor unit and is displayed on the computer monitor. The change in enthalpy is displayed as a signal deviating from the baseline.

2.5 Experimental protocol

2.5.1 Preparation of the liposomes

There exist several methods of producing liposomes. These include extrusion, “reversed phase evaporation”, by means of ultrasound, high pressure homogenising and mechanical dispersion (New, 1990; Lascic, 1993; Burger and Wachter, 1993).

The mechanical dispersion of lipids is a simple and effective method used for producing liposomes for experimental purposes. This was the method used in this work and has been described previously (Kursch et al., 1983; Hanpft and Mohr, 1985). As stated above (section 2.1.1), the sodium salt of DPPA and DPPC were obtained from Sigma-Aldrich Chemie (Schnelldorf, Germany). These were used without further purification.

In a glass vial, appropriate amounts of test substance dissolved in chloroform or in a 15:2, v/v chloroform/methanol mixture or dimethyl sulfoxide (DMSO purity over 99,5%) as solvent were added to 5mg phospholipids (200µl of a 2.5% solution in chloroform as solvent in the case of DPPC). DPPA is virtually insoluble in chloroform as well as in other current commonly used solvents including DMSO. Consequently, DPPA was measured directly into the vials as a dry substance and the calculated appropriate amounts of substance added. The solvent was evaporated overnight using a rotary vacuum evaporator (SC440 Speed Vac® Concentrator, Savant Instruments Inc., Holbrook, USA). With DMSO present as a component of the solvent, the drying was carried out at a temperature of 43°C (medium heating setting on the device) otherwise the temperature setting was ambient (lowest setting on the device).

100µl of a 14mM TES-histidine buffer solution, adjusted to a pH value of 6 using HCl was then pipetted into the vials. This resulted in dispersions containing 68mmol of DPPC and 75mmol of DPPA per litre of buffer respectively. The molarity of the test substances in the liposome dispersion measured varied between $6x10^{-4}$M and 0.2M depending on the solubility of the substance. The values are given in this work as fractions of the quantity of phospholipid.

The resulting dispersions were then heated for 2 hours in the now stoppered glass vials at a temperature higher than the phase transition temperature of the used phospholipid. For DPPA, the temperature was set at 73°C and for DPPC 53°C. During this period, the vials were subject to vigorous mechanical agitation on a mixer at definite intervals in a time progressive manner, based on the following scheme: the

shaking was done immediately before heating began and again after 10, 30, 60, 90 and finally 120 minutes respectively after beginning the heating process, the agitation period always lasting 10 seconds. Through this procedure, depending on the molar ratio involved, multilamellar liposomes of different sizes were formed, with the latter clearly visible under a light microscope.

About 10µl of the respective suspensions was weighed into an aluminium DSC pan, the latter sealed and the contents measured using calorimetry. Chapman et al. observed slight variations in the phase transition temperature depending on water content of the liposome dispersions (Chapman and Urbina, 1974). Cevc et al. also found a dependence of the T_t of acidic phospholipids on pH (Cevc et al., 1981). Thus, these factors were kept constant throughout the measurements carried out in this work to rule out errors that could result from varying these quantities.

2.5.2 Measuring procedure

The computer and DSC were turned on, and the DSC program, Pyris, started. The sealed pan containing the weighed sample (to a tenth of a milligram) and the reference were then loaded into the specimen-holders of the calorimeter. The run parameters, temperature range and rate of heating, were programmed into the DSC and the run started.

2.5.2.1 Instrument settings.

The peak size and shape are generally influenced by the following factors: sample weight, heating rate, purge gas and purge gas flow rate, initial and final heating temperature, DSC pan used and the reference substance. These factors were therefore kept constant during the measurements. Generally, the measured heat flow is related to the heating rate to an extent. Very high heating rates reduce resolution and cause a melting together of adjacent lying peaks, leading to difficulties in their separation (Ehrenstein et al., 1998).

The following settings were chosen empirically.

Heating rate: $5°CMin^{-1}$

Cooling rate: $15°CMin^{-1}$

Increasing the heating rate in the measurements carried out in this work to a value of $15°CMin^{-1}$ did not bring about any significant differences in the signals observed. However, the heating rate was kept at $5°CMin^{-1}$ for all measurements carried out.

The purge gas used was nitrogen and the purge gas flow rate $20mlMin^{-1}$

Data was collected throughout the run by the computer and plotted in real time or against temperature on the monitor.

The investigated temperature ranges were as follows:

For measurements involving DPPC-containing samples: between -10 and 55°C.

For measurements involving DPPA-containing samples: between -10 and 75°C.

Some sources in the literature mention an empty sealed pan as the reference (Ehrenstein et al., 1998; Heller, 2000) although it may be the buffer solution used in preparing the liposome dispersion. A test with both buffer and an empty pan revealed that the signal positions are not affected. They remain same in either case. The only difference observed in the thermograms was the horizontal position of the baselines, obtained from the heating and cooling process, relative to one another. The explanation for this is that as long as there is no transition, the relative heat flow through both pans remains constant. The results achieved are therefore not affected, provided there is consistency in the reference used. In any case, buffer solution was used as reference in this work. Measurements were carried out during the heating as well as the cooling process, but the heating curves were used for evaluation purposes since this involves a melting process. Cooling was achieved using liquid nitrogen. In order to prevent the condensation of water vapour or ice formation in the measuring chambers that can result due to the conditions prevailing in these chambers, the latter were flushed with pressurised dry nitrogen during operation.

2.5.3 Data evaluation and interpretation

Using the calibration curve that is stored in the microprocessor unit in the device, the temperature differential between the sample and the reference is converted to heat units. The heat value is next converted to power by dividing the heat by the run time. This plotted against temperature constitutes the DSC thermogram. The interpretation of the thermogram is generally straightforward; in this case, an endothermic process is shown as an upward deviation (observed on heating) while an exothermic process is shown as a downward deviation in the baseline and is evident in the cooling process.

A quantitative assessment of the heat uptake through direct comparison of the achieved peak areas among different substances would normally require the knowledge of the quantity of test substance in the sample being investigated. This can always be calculated from the weight of the sample.

2.5.4 Statistical analysis

The average values of phase transition temperatures, T_t, and the resultant changes in transition temperature caused by a test substance, ΔT_t, given in this work are the arithmetic means and these were calculated based on the following equation:

$$\bar{x} = \frac{1}{n}\sum_{1=n}^{n} x_i = \frac{1}{n} \cdot \left(x_1 + x_2 + x_3 + ... + x_n\right) \qquad \text{Equation 3}$$

$\bar{x}$ is the mean value

$x_1, x_2, \ldots, x_n$ are the individual measured values

n is the number of individual values (sample size)

The Standard Error of the Mean (SEM), a measure of how far the sample mean is likely to be from the true population mean was calculated based on the following equation:

$$SEM = \frac{SD}{\sqrt{n}} \qquad \text{Equation 4}$$

3. Results

3.1 Characterisation of the interaction of model substances with phospholipid membranes

As explained in the introduction, one of the major objectives of this work was the investigation of the properties of muscarinic acetylcholine receptor ligands among other test substances and the characterisation of the nature of their interactions with certain cellular components using the physicochemical properties resulting from interactions with phospholipid bilayers. The major property used in this characterisation was the phase transition temperature, T_t of liposomes. The signal obtained from experiments measuring this property varied with the former being unique for the substance being investigated. The drug induced signal differed in shape and size from the signal obtained from experiments with liposomes containing pure phospholipid depending on the nature and extent of the interaction with the phospholipid bilayers.

3.1.1 Thermograms obtained from experiments with pure phospholipid liposomes

3.1.1.1 DPPC-liposomes

As observed in previous experiments (Cater et al., 1974; Papahadjopoulos et al., 1975; Frenzel et al., 1978), the phase transition signal of liposomes containing solely DPPC in aqueous solution comprises two transition peaks (Fig. 3-1): a pre-transition, and a main transition peak. The pre-transition peak is much smaller and is clearly set apart from the main transition peak. The measured average onset temperature of the pre-transition peaks in this work was 36°C while that of the main transition peak was 42°C (Tab. 1). These values are consistent with those obtained by other groups that generally lay between 41 and 42°C. The pre-transition peak usually had a broader base than the more spiky main transition peak. While the pre-transition peak rose gently from the baseline to its maximum value, this was not the case with the main transition peak, with the beginning of the peak leaving the baseline much more abruptly. This facilitated the determination of the onset temperature. The descending arm of the peak was gentler, with the whole base of the peak not extending over a range of 2.5 degrees. In all, the slopes on both sides of the main transition peak were much steeper than those of the pre-transition peak. These findings are illustrated in the

signal shown in Fig. 3-1, representative of 30 experiments conducted with liposome dispersions containing pure DPPC.

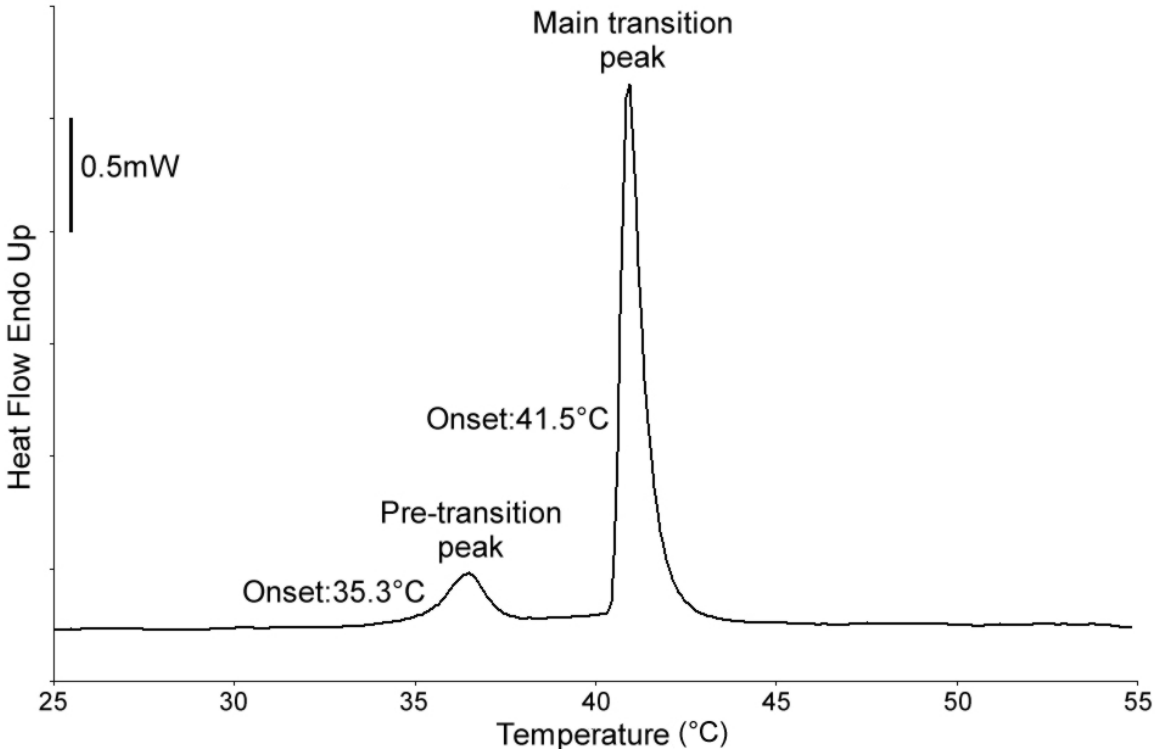


Figure 3-1: Shown is an original DSC signal obtained from the measurement of pure DPPC liposomes at a heating rate of 5°CMin^{-1}. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

Table 3-1 shows the average onset values of the transition temperatures of the pre- and the main transition peaks.

Substance	T_p	ΔH	n	T_t	ΔH	n
DPPC	36.1 ± 0.23	0.25	30	41.5 ± 0.21	1.74 ± 0.05	30
DPPA	-	-	-	63.4 ± 0.15	2.04 ± 0.07	35

Table 3-1: Onset values of the pre-transition and main transition temperatures ± standard errors of the mean obtained from measurements using liposomes containing DPPC and DPPA as sole component in aqueous solution. T_p: onset temperature of the pre-transition peak. T_t: onset temperature of the main peak. (ΔH: Enthalpy change of transition)

3.1.1.2 DPPA liposomes

Contrary to signals resulting from experiments carried out with DPPC liposomes, signals from pure DPPA liposomes only possess one peak as can be seen in the thermogram below. Here, the peak ascends initially much more gradually, compared with the main transition signals obtained from DPPC measurements. The descending slope is much steeper. All experimental conditions were kept constant throughout the measurements with DPPC as well as DPPA liposomes.

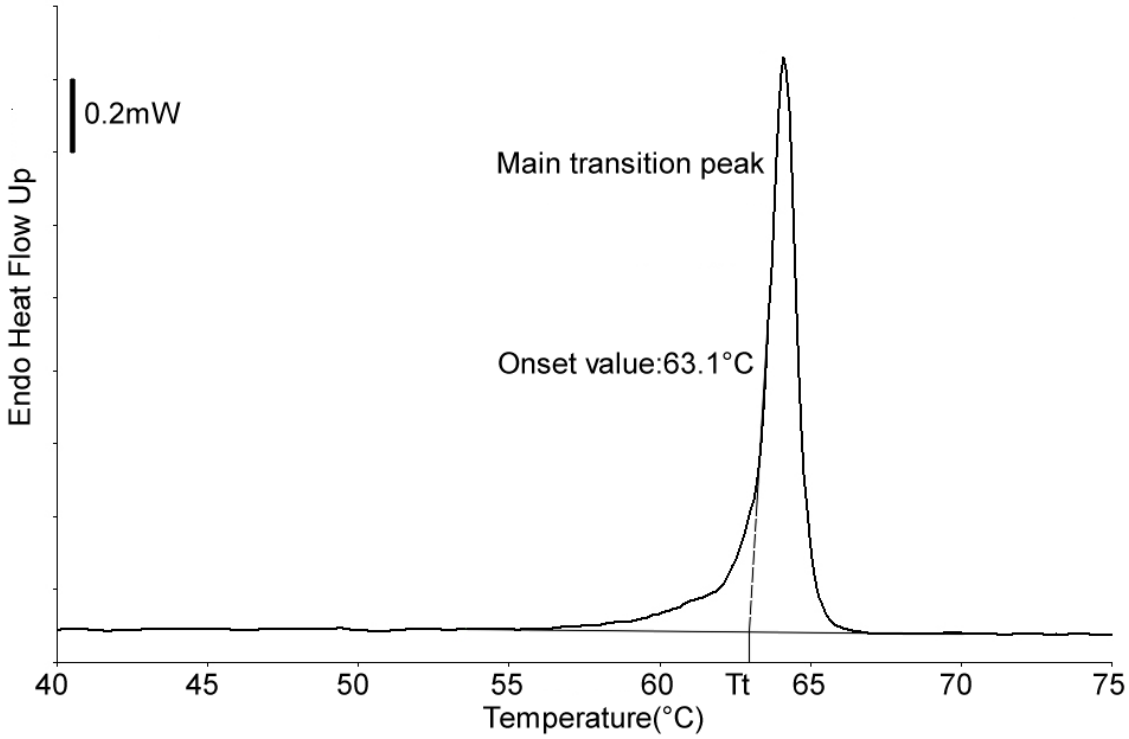


Figure 3-2: DSC signal obtained from the measurement of DPPA liposomes prepared in the absence of test substance. A pre-transition signal is absent here. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

The average value of the onset temperature, T_t, obtained from 35 experiments was 63.4 ± 0.15 where 0.15 represents the standard error of the mean.

Due to the shape of the peaks, the average enthalpy change for the DPPA signal turned out to be greater than that from the DPPC peaks. Table 3-1 in the previous section summarises these findings.

3.2 Thermograms obtained from experiments with test substances

3.2.1 Propranolol

The β-adrenoceptor blocker propranolol has been investigated in similar experiments by other groups earlier (Hanpft and Mohr, 1985; Mohr, 1987). A comparison of T_t with those obtained here resulting in similar values would further strengthen the validity of the method. Results obtained from measuring DPPA liposomes containing propranolol in various molar ratios are shown in the original thermograms in the figure below.

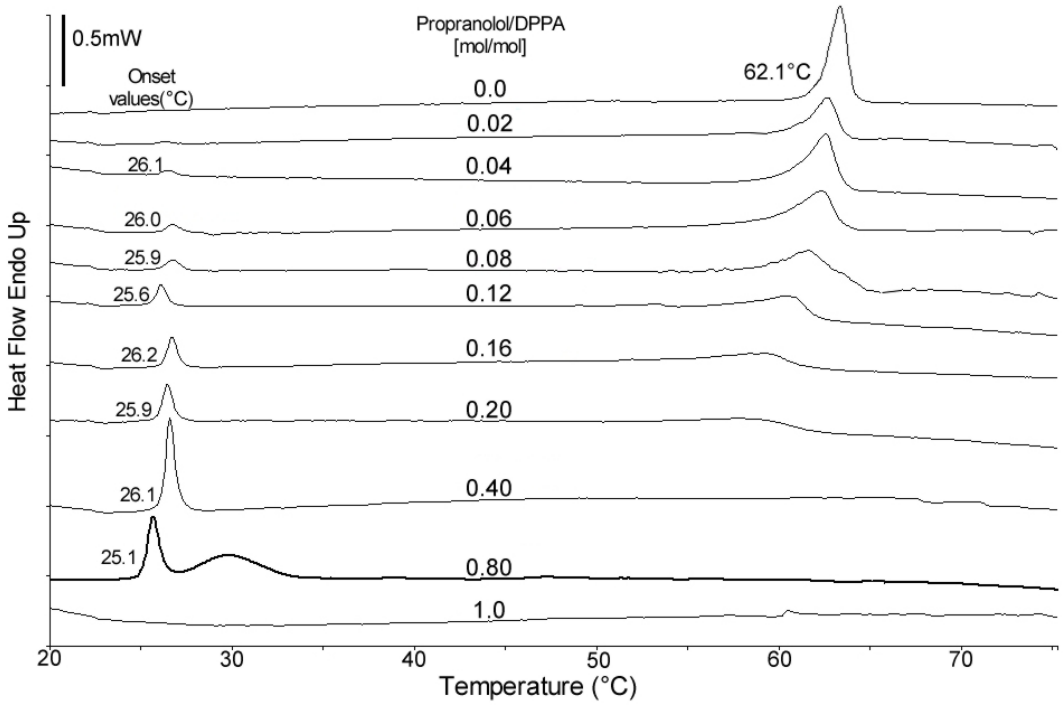


Figure 3-3: Thermograms obtained from experiments carried out using liposomes containing DPPA with the β-adrenoceptor blocker propranolol as test substance.
Ordinate: endothermic heat flow. ***Abscissa***: sample temperature in °C. Shown here are the results from one of three experiments.

A substance-induced peak, however small, is already recognisable at a molar ratio of propranolol to DPPA of 0.02. This peak becomes narrower with increasing molar ratios. Results obtained earlier (Hanpft and Mohr, 1985; Mohr, 1987) showed a substance-induced peak with onset value at 27°C. The similarity with the values obtained here is evident.

3.2.2 Phenylpropylamines

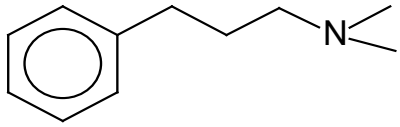
3.2.2.1 DPPA liposomes

The transition temperatures were affected to various extents by the phenylpropylamines investigated. The reduction with DPPA is more or less constant and the change of T_t (ΔT_t) is usually a fixed value evident in peaks that emerge beyond a certain molar ratio.

A measure of the lipophilicity of the compounds is given in terms of the logarithm of the octanol/buffer partition coefficient (log P) of the substances. A direct comparison can be made between the extent to which the substances reduce the phase transition temperature and these coefficients. However, since this was not the prior objective of this work, details of these comparisons were not pursued any further. The log P values given for the various substances were determined by C. Klein using High-Performance Liquid Chromatography (HPLC) and a pH value of 7.4. M. Klingmüller using the method of shake-flask octanol/buffer partitioning determined those marked with an asterisk.

The thermograms shown in each diagram result from individual measurements from the same experiment and have been compiled on single graphs for clarity.

3.2.2.1.1 Experiments containing KH210 (N,N-dimethyl-3-phenylpropylamine) as test substance



This simple planar parent compound with a relatively low log P value (1.50), compared with the other phenylpropylamines, brought about a clearly observable reduction in the phase transition temperature. At the temperatures at which the liposome dispersions were prepared, the dispersions resulting from low substance/phospholipid molar ratios appeared translucent light-white and gradually became milky white with increasing molar ratios. The viscosity and colour intensity increased on cooling to room temperature.

Figure 3-4 on the next page shows the results of measurements of these liposome dispersions.

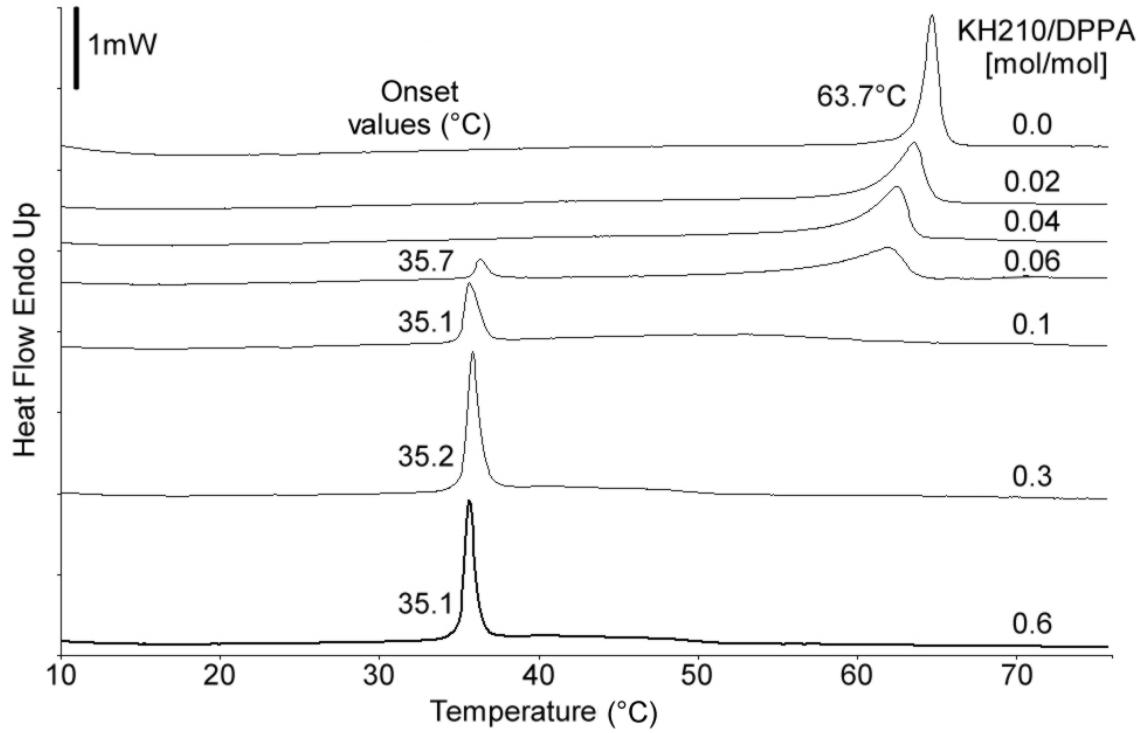


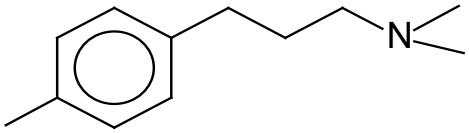
Figure 3-4: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH210 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

As can be seen on the graph, the measurements yielded thermograms that showed a temperature reduction of about 28.5°C. There is an initial gradual reduction in size and a shift towards lower temperatures of the original signal with its eventual disappearance as the substance-induced signal emerges and grows larger. This was typical of measurements carried out with DPPA. The results are summarised in the table below.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t
0.06	28.0	0.05	28.2
0.1	28.6	0.1	28.8
0.3	28.5	0.3	29.0
0.6	28.6	0.6	28.8
Average ΔT_t	28.4 ± 0.14	Average ΔT_t	28.7 ± 0.17

Table 3-2: DPPA phase transition temperature reduction by KH210. The average value of ΔT_t from all the measurements (n = 8) amounted to 28.6 ± 0.10°C (mean value ± SEM). SEM is the standard error of the mean.

3.2.2.1.2 KH211 (N,N-dimethyl-4'-methyl-3-phenylpropylamine)



As is evident from the above structure, the compound is a simple derivative of the parent compound KH210, with a methyl group brought unto the para-position of the benzene ring. This leads to a marginal increase in the length of the compound compared to that of the parent compound. It also brings about an increase in the lipophilicity, with the log P value rising from 1.50 for KH210 to 2.00. Original thermograms from experiments with this compound are shown in the figure below. The difference in the reduction of the phase transition temperature compared to that caused by the parent compound is not negligible.

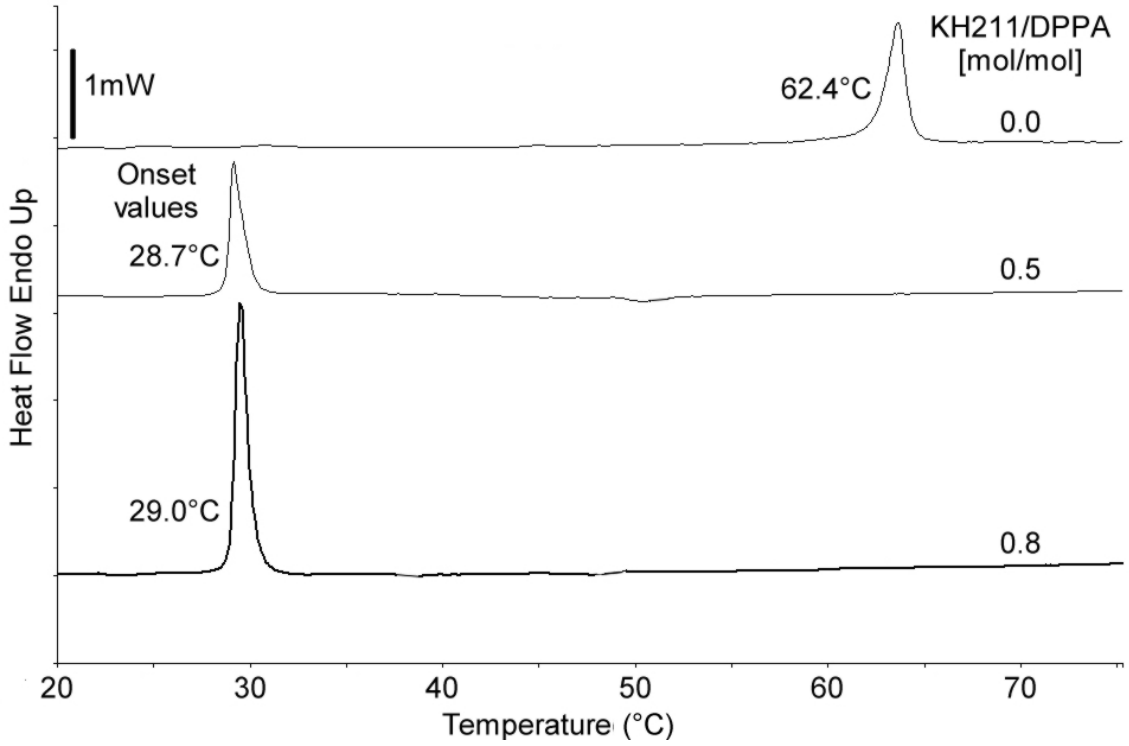


Figure 3-5: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH211 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

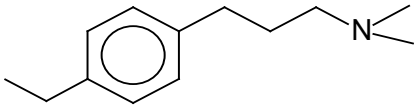
As can be seen in the two preceding figures, the substance induced phase transition temperature remained relatively constant beyond a certain molar ratio through the entire measured range. Hence in the figures that follow with phenylpropylamines as test substances, only three of four molar ratios are typically shown with thermograms resulting from molar ratios within the measurable range. Beyond a certain molar ratio

which differed for each substance, no peaks could be observed due to a detergent effect that results from the high concentration of the test substance, a phenomenon that has previously been discussed (Hanpft and Mohr, 1985). At this molar ratio and beyond, the dispersion became clear and colourless and the consistence changed with the viscosity dropping.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.1	32.8	0.5	33.7
0.2	32.5	0.8	33.4
0.6	32.4		
1.0	32.0		
Average ΔT_t	32.4 ± 0.17	Average ΔT_t	33.6 ± 0.40

Table 3-3: DPPA phase transition temperature reduction by KH211. The average value of ΔT_t from all the measurements (n = 6) amounted to 32.8 ± 0.26°C (mean value ± SEM).

3.2.2.1.3 KH212 (N,N-dimethyl-4'-ethyl-3-phenylpropylamine)



An extension of the length of the substituent by a methylene unit in para position results in the compound KH212. This extension in length brings about an increase in the log P value to 2.45 as well as a further increase in the phase transition temperature reduction, although the absolute increase does not have the same value as that brought about by introducing a methyl group in the parent compound (see above). This is shown in the thermograms from an experiment performed using KH212.

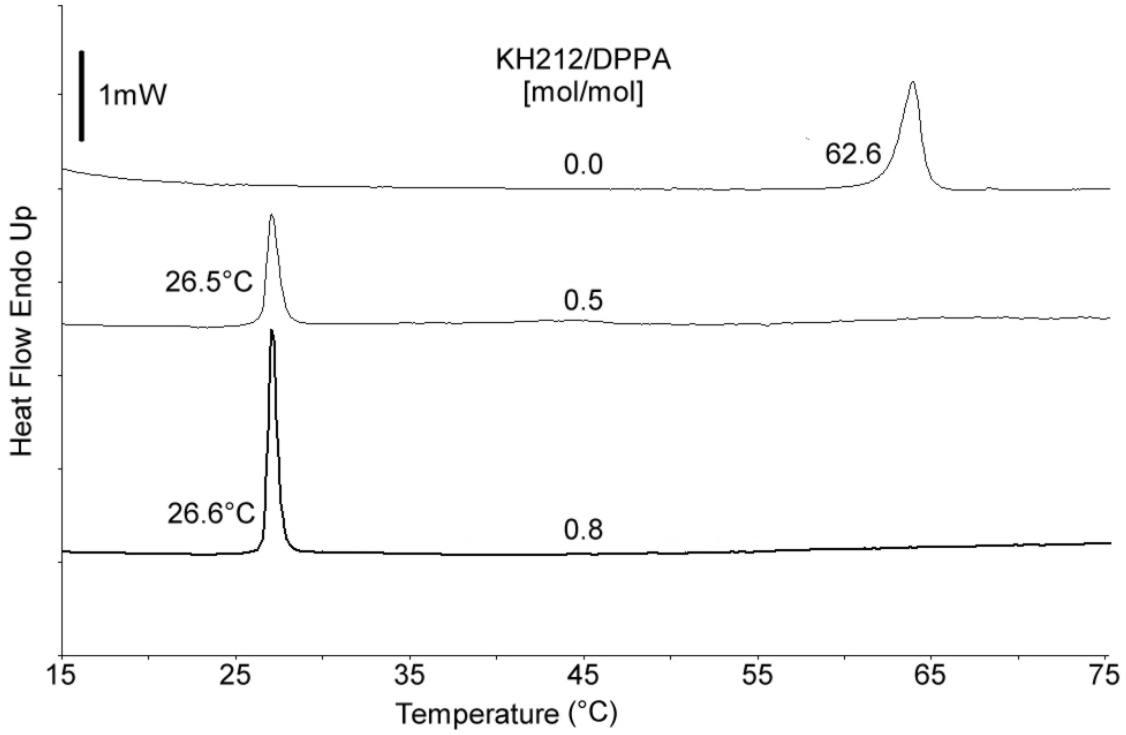
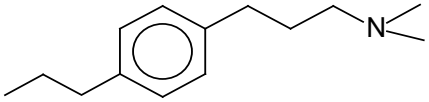


Figure 3-6: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH212 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.2	36.1	0.5	36.1
0.4	36.0	0.8	36.0
0.6	35.2		
1.0	35.0		
Average ΔT_t	35.6 ± 0.28	Average ΔT_t	36.1 ± 0.05

Table 3-4: DPPA phase transition temperature reduction by KH212. The average value of ΔT_t from all the measurements (n = 6) amounted to 35.7 ± 0.20°C (mean value ± SEM).

3.2.2.1.4 KH213 (N,N-dimethyl-4'-*n*-propylphenylpropylamine)



As well as increasing the length and the log P value (2.89), an *n*-propyl rest as substituent increases the reduction in phase transition even further. However, the peak tends to get smaller at a molar ratio of substance to DPPA to 0.8. The dispersions got more viscous with increasing molar ratio of substance to phospholipid.

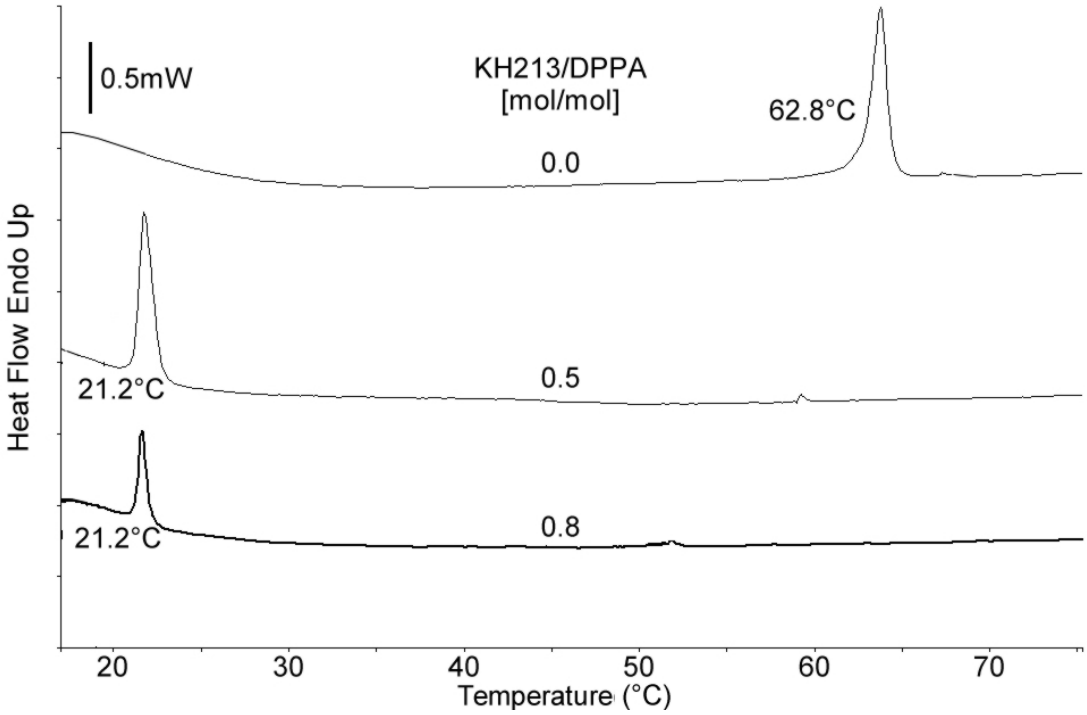
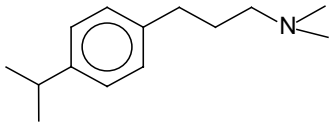


Figure 3-7: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH213 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 0.5mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.1	41.3	0.5	41.6
0.2	40.2	0.8	41.6
0.4	39.9		
0.6	40.7		
0.8	40.8		
Average ΔT_t	40.6 ± 0.24	Average ΔT_t	41.6 ± 0.0

Table 3-5: DPPA phase transition temperature reduction by KH213. The average value of ΔT_t from all the measurements (n = 7) amounted to 40.9 ± 0.25°C (mean value ± SEM).

3.2.2.1.5 KH214 (N,N-dimethyl-4'-*iso*-propyl-propylamine)



An isopropyl rest was incorporated into the parent compound, the molecule becoming shorter than KH213 and also having a reduced log P value (2.75) in comparison. Results of DSC measurements using this compound are shown in the figure below.

Like with the previous substances, here too, the dispersion got more viscous with increasing substance to phospholipid molar ratio.

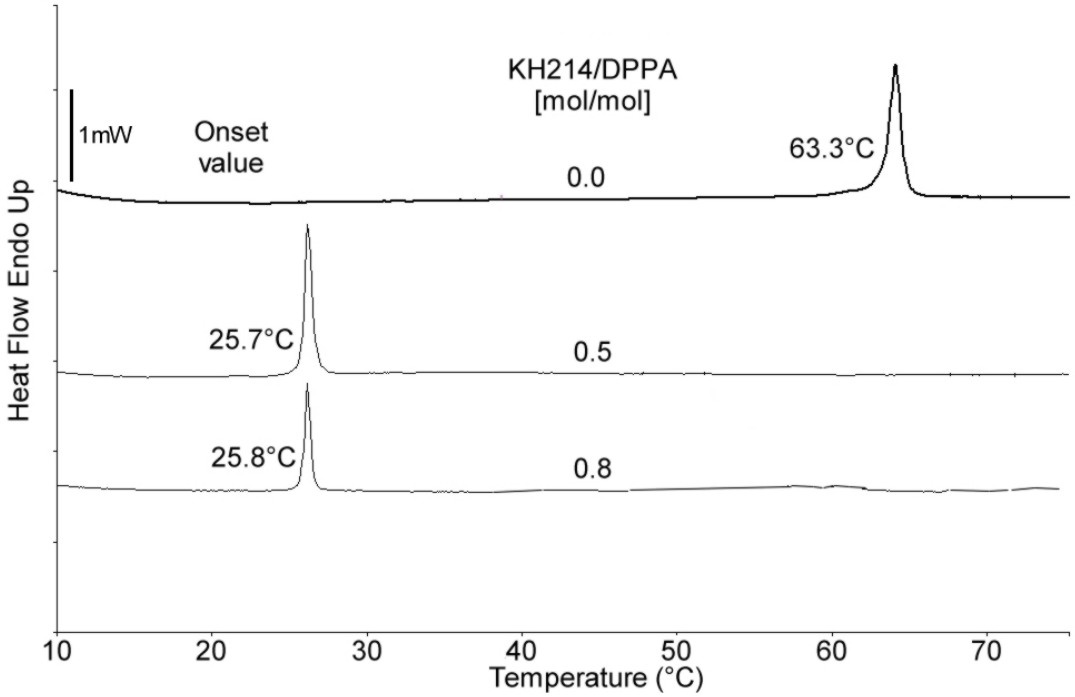
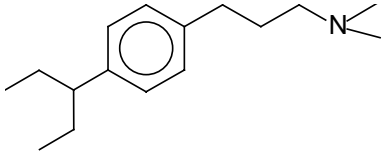


Figure 3-8: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH214 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.05	37.1	0.5	37.6
0.1	36.9	0.8	37.5
0.4	35.9		
0.6	35.7		
Average ΔT_t	36.4 ± 0.35	Average ΔT_t	37.6 ± 0.05

Table 3-6: DPPA phase transition temperature reduction by KH214. The average value of ΔT_t from all the measurements (n = 6) amounted to 36.8 ± 0.33°C (mean value ± SEM).

3.2.2.1.6 CK19 (N,N-dimethyl-3', p-pentylphenylpropylamine)



The substituent in this case does not differ in length from the n-propyl moiety in KH213 (see above). Remarkably, the phase transition temperature of DPPA was reduced by CK19 and KH213 to the same extent as is shown below. But the octanol/buffer partition coefficients of the two substances differ. Whereas KH213 has a log P value of 2.89, that of CK19 is 3.68.

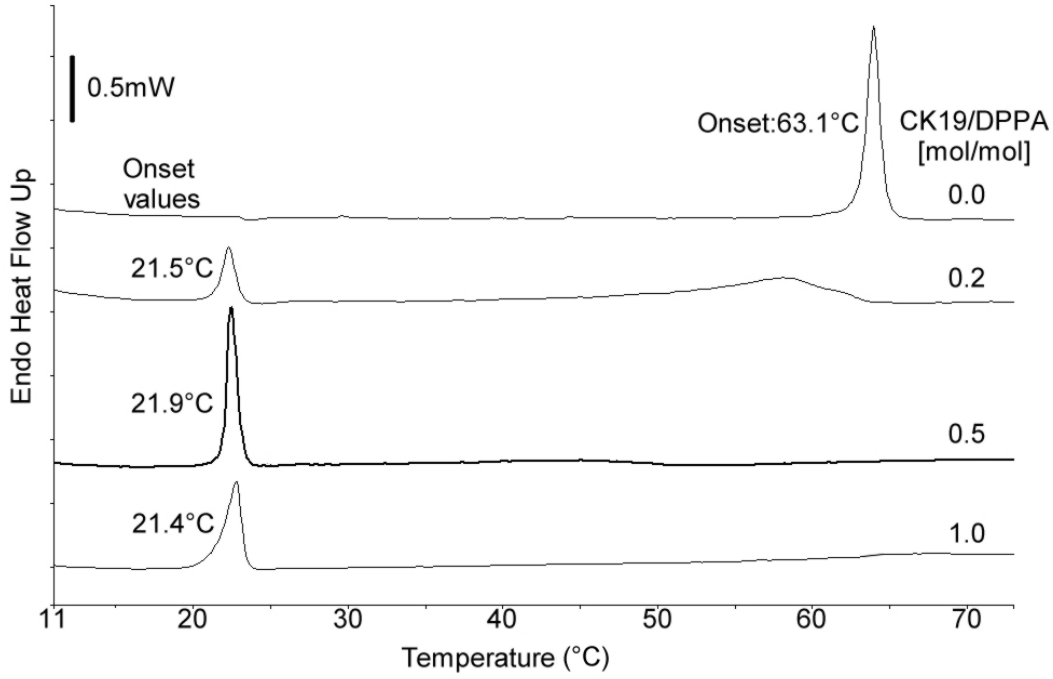
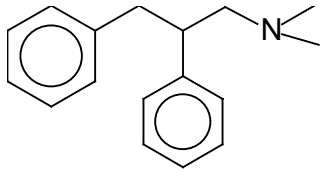


Figure 3-9: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance CK19 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 0.5mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.1	39.0	0.2	41.6
0.2	41.9	0.5	41.2
0.4	39.9	1.0	41.7
0.6	40.8		
Average ΔT_t	40.4 ± 0.62°C	Average ΔT_t	41.5 ± 0.15°C

Table 3-7: DPPA phase transition temperature reduction by CK19. The average value of ΔT_t from all the measurements (n = 7) amounted to 40.9 ± 0.04°C (mean value ± SEM).

3.2.2.1.7 CK84 (N,N-dimethyl-2,3-diphenylpropylamine)



Here, the substituent is not affixed unto the phenyl ring but directly on the propyl chain. Resultant thermograms from an experiment using this substance are shown below. The dispersion also became very viscous at the molar ratio of 0.8. The substance has a log P value of 2.27.

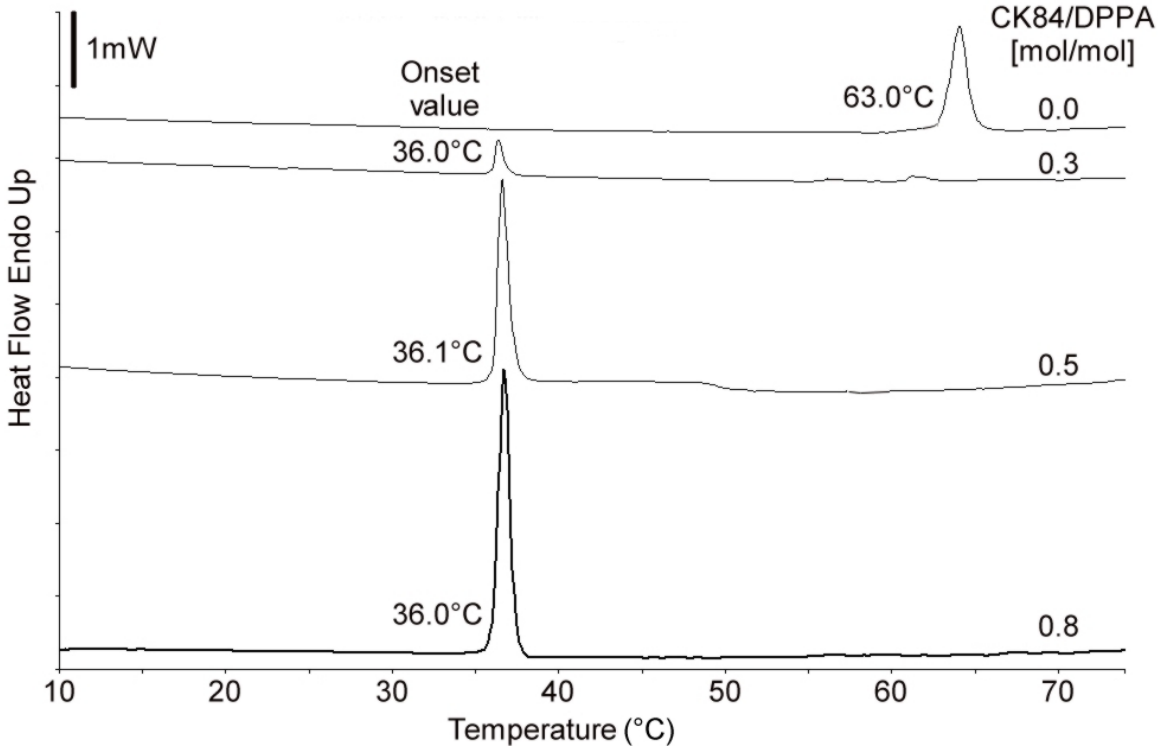
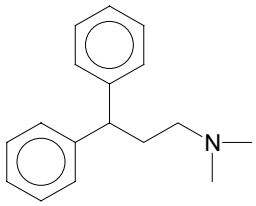


Figure 3-10: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance CK84 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.3	27.0	0.5	27.3
0.5	26.9	0.8	27.2
0.8	27.0		
Average ΔT_t	27.0 ± 0.03°C	Average ΔT_t	27.3 ± 0.05

Table 3-8: DPPA phase transition temperature reduction by CK84. The average value of ΔT_t from all the measurements (n = 5) amounted to 27.1 ± 0.07°C (mean value ± SEM).

3.2.2.1.8 CK94 (N,N-dimethyl-3,3-diphenylpropylamine)



In CK94, the substituent is on the gamma carbon atom of the propyl chain. Here also, like with CK84 there is no gain in length compared to the original compound KH210. The log P value was a little higher, amounting to 2.35. At the molar ratio of 0.8, the dispersion became more or less “saturated”, with the precipitation of substance on the internal surface of the glass vials.

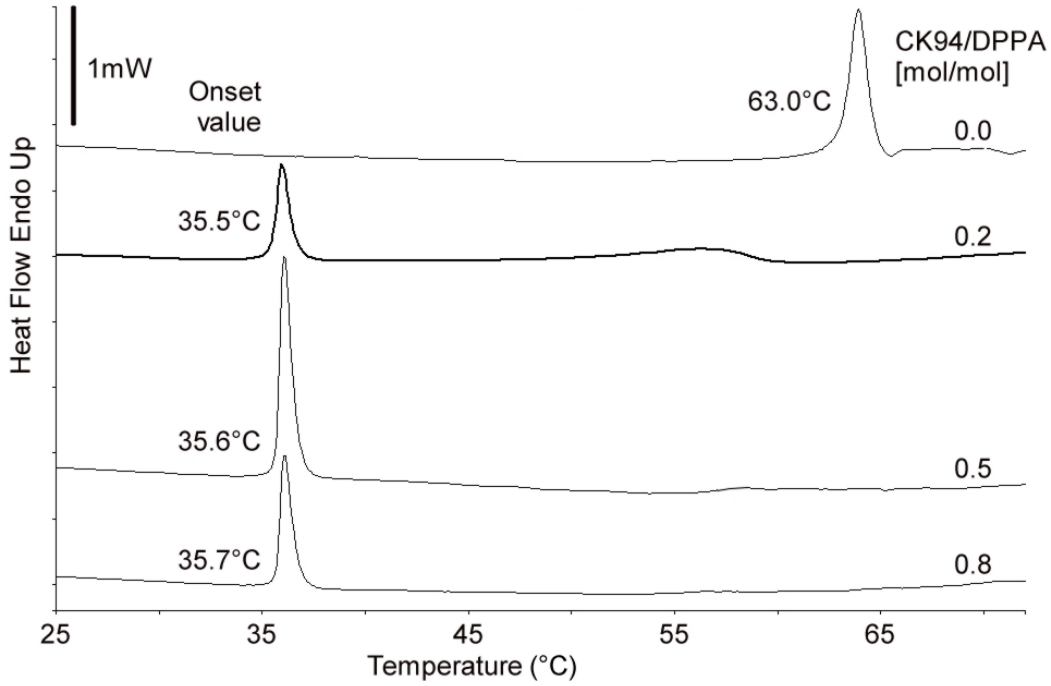
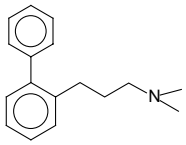


Figure 3-11: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance CK94 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 0.5mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.2	27.5	0.3	27.2
0.5	27.4	0.5	27.7
0.8	27.3	0.8	27.8
Average ΔT_t	27.4 ± 0.06°C	Average ΔT_t	27.5 ± 0.19

Table 3-9: DPPA phase transition temperature reduction by CK94. The average value of ΔT_t from all the measurements (n = 6) amounted to 27.5 ± 0.09°C (mean value ± SEM).

3.2.2.1.9 CK53 (N,N-dimethyl-3,2'-biphenylpropylamine)



Here, the phenyl substituent is situated in the ortho-position. The absolute length of the molecule does not increase with respect to the parent compound, however, the spatial arrangement of both molecules differ. With a log P of 2.72, the substance has a value higher than the 1.50 of the parent compound. Thermograms from a representative experiment carried out with the substance are shown in the figure below. Despite the lower lipophilicity, the compound probably penetrates deeper into the bilayer, bringing about a reduction in T_t greater than that of the parent compound.

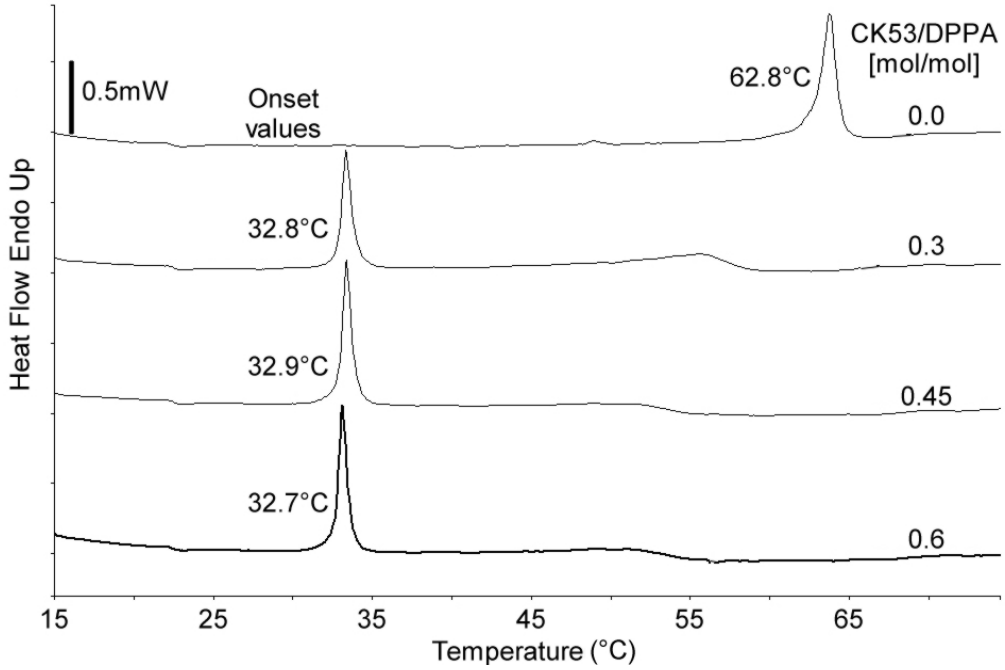
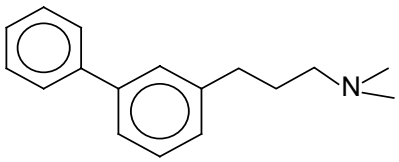


Figure 3-12: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance CK53 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 0.5mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.3	30.0	0.1	29.2
0.5	29.9	0.3	30.5
0.6	30.1	0.5	28.6
		0.8	30.3
Average ΔT_t	30.0 ± 0.06°C	Average ΔT_t	29.7 ± 0.40°C

Table 3-10: DPPA phase transition temperature reduction by CK53. The average value of ΔT_t from all the measurements (n = 7) amounted to 29.8 ± 0.22°C (mean value ± SEM).

3.2.2.1.10 CK92 (N,N-dimethyl-2',3-biphenylpropylamine)



Unlike the preceding compound, CK53, this compound has a phenyl substituent in the meta position. Accordingly, the length of the compound is increased compared to CK53, and the log P value too: 3.03. Thermograms from an experiment using CK92 are shown in the figure below.

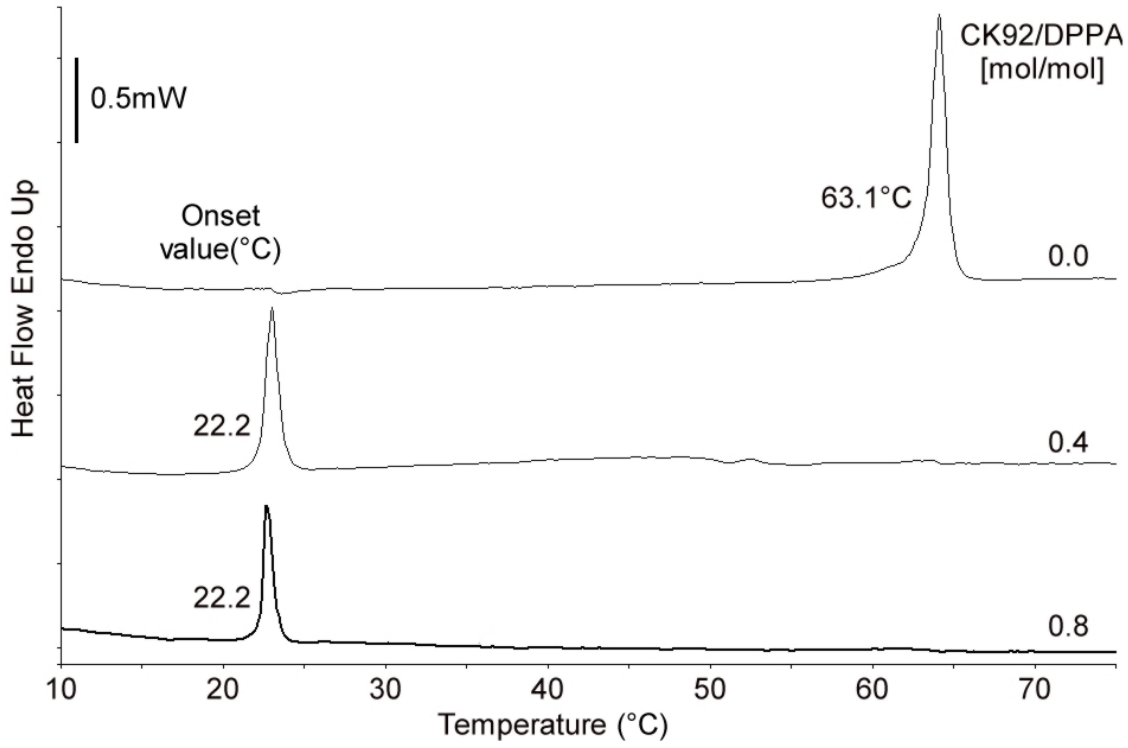
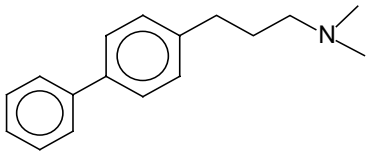


Figure 3-13: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance CK92 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 0.5mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.4	40.9	0.3	42.3
0.8	40.9	0.5	41.6
		0.8	41.4
Average ΔT_t	40.9 ± 0.0	Average ΔT_t	41.8 ± 0.27

Table 3-11: DPPA phase transition temperature reduction by CK92. The average value of ΔT_t from all the measurements (n = 7) amounted to 41.4 ± 0.26°C (mean value ± SEM).

3.2.2.1.11 KH204 (N,N-dimethyl-3,4'-biphenylpropylamine)



Signals obtained from a set of experiments using the substance KH204 and DPPA are shown in the graph below. The substance is longest so far and reduces T_t the most. This is accompanied by a large value for the octanol/buffer coefficient, a log P of 3.16.

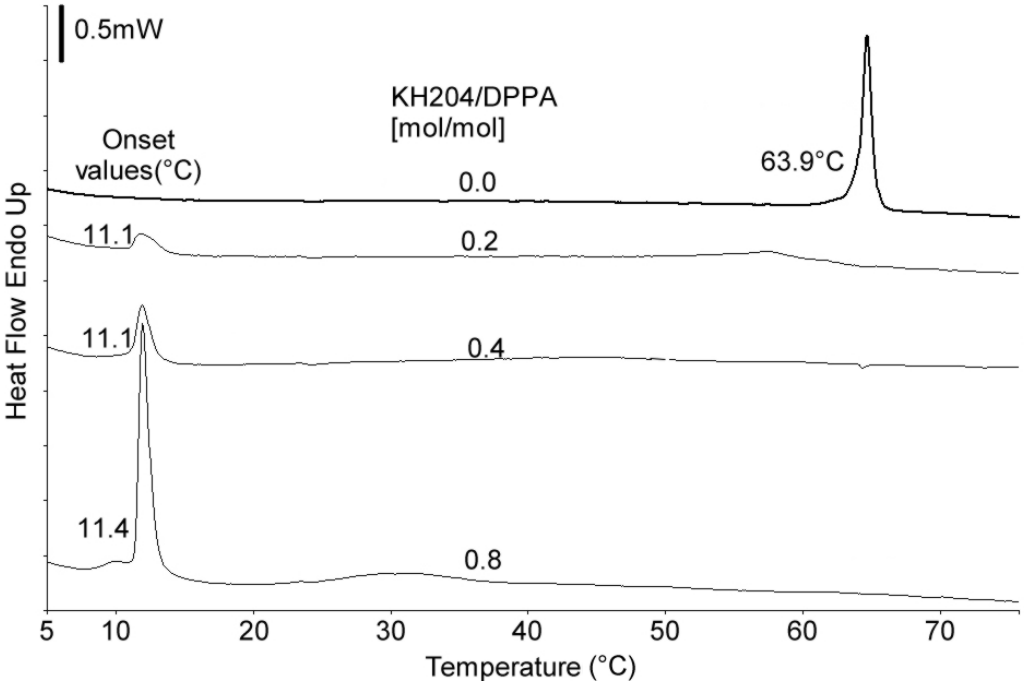
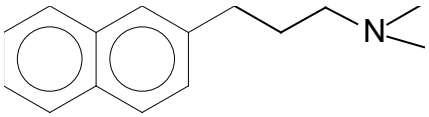


Figure 3-14: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH204 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 0.5mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.16	53.3	0.2	52.8
0.2	53.7	0.4	52.8
0.4	53.4	0.8	52.5
0.8	53.2		
Average ΔT_t	53.4 ± 0.11°C	Average ΔT_t	52.7 ± 0.10°C

Table 3-12: DPPA phase transition temperature reduction by KH204. The average value of ΔT_t from all the measurements (n = 7) amounted to 53.1 ± 0.16°C (mean value ± SEM).

3.2.2.1.12 KH220 (N,N-dimethyl-3-naphthylpropylamine)



The naphthyl substituent is planar and the substance has a log P value of 2.62*. Its presence in the liposome dispersion affects the phase transition temperature as shown below.

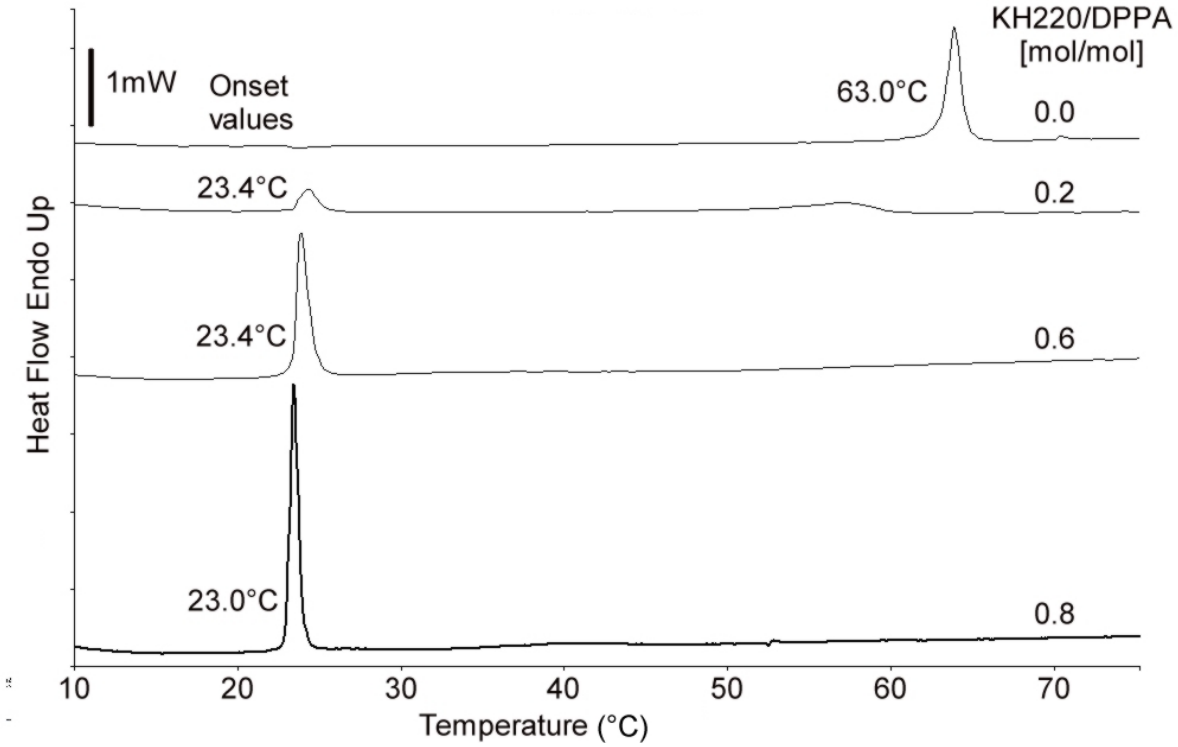


Figure 3-15: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH220 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

Experiments conducted					
1		2		3	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.05	40.4	0.5	40.5	0.2	39.6
0.1	41.2	0.8	40.5	0.6	39.6
0.2	39.2			0.8	40.0
0.4	39.2				
0.6	39.1				
Average ΔT_t	39.8 ± 0.42	Average ΔT_t	40.5 ± 0.05	Average ΔT_t	39.7 ± 0.13

Table 3-13: DPPA phase transition temperature reduction by KH220. The average value of ΔT_t from all the measurements (n = 10) amounted to 39.9 ± 0.22°C (mean value ± SEM).

3.2.2.1.13 KH216 (N,N-dimethyl-4'-methoxyphenylpropylamine)

Due to the presence of a methoxy group in the para position, this derivative would be expected to act differently from the methyl-containing substance KH211, caused by the electonegativity and size of the oxygen atom present in the compound, and also the inductive effect it has on the benzene ring. But a look at the figures from both substances does not seem to show any significant difference in the onset temperatures. The substances, however, do differ in their octanol/buffer partition coefficients. KH216 has a log P value of 1.48. KH211 had a value of 2.00.

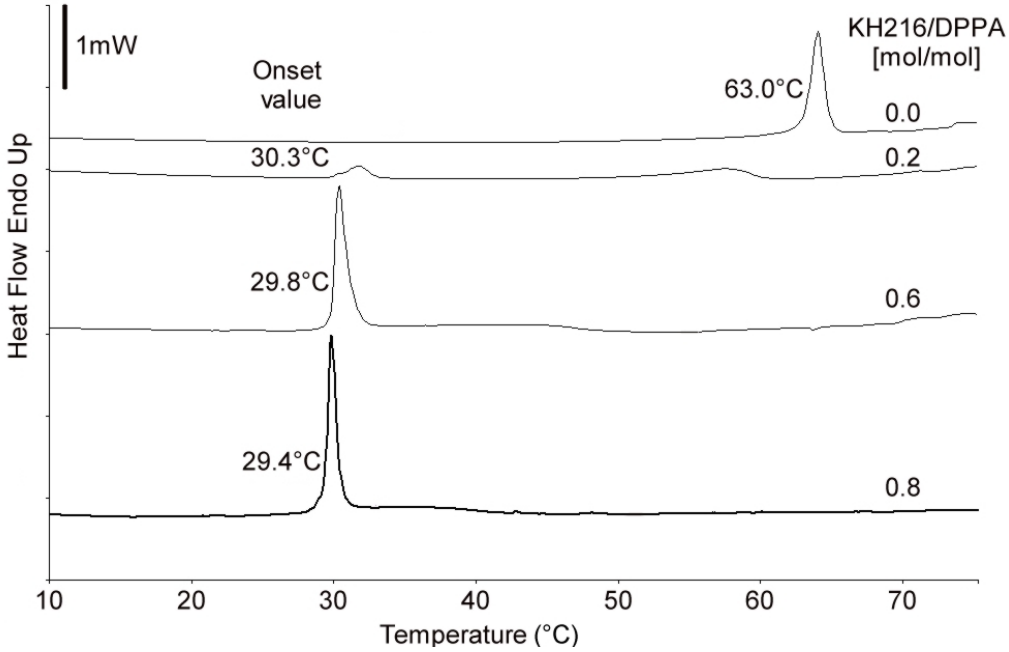
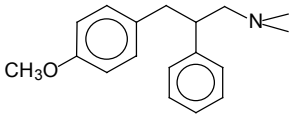


Figure 3-16: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH216 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

Experiments conducted					
1		2		3	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.1	32.7	0.2	32.7	0.5	33.0
0.2	32.2	0.6	33.2	0.8	33.1
0.4	32.5	0.8	33.6		
0.6	32.5				
1.0	32.6				
Average ΔT_t	32.5 ± 0.84	Average ΔT_t	31.2 ± 0.26	Average ΔT_t	33.1 ± 0.06

Table 3-14: DPPA phase transition temperature reduction by KH216. The average value of ΔT_t from all the measurements (n = 10) amounted to 32.8 ± 0.13°C (mean value ± SEM).

3.2.2.1.14 CK41 (N,N-dimethyl-3[4'-methoxyphenyl]-2-phenylpropylamine)



This compound is a derivative of CK84 containing a methoxy group as shown in the structural formula above. Unfortunately, the octanol/buffer partition coefficient for this compound was not determined. The results of an experiment carried out with this substance in DPPA liposomes are shown in the figure below.

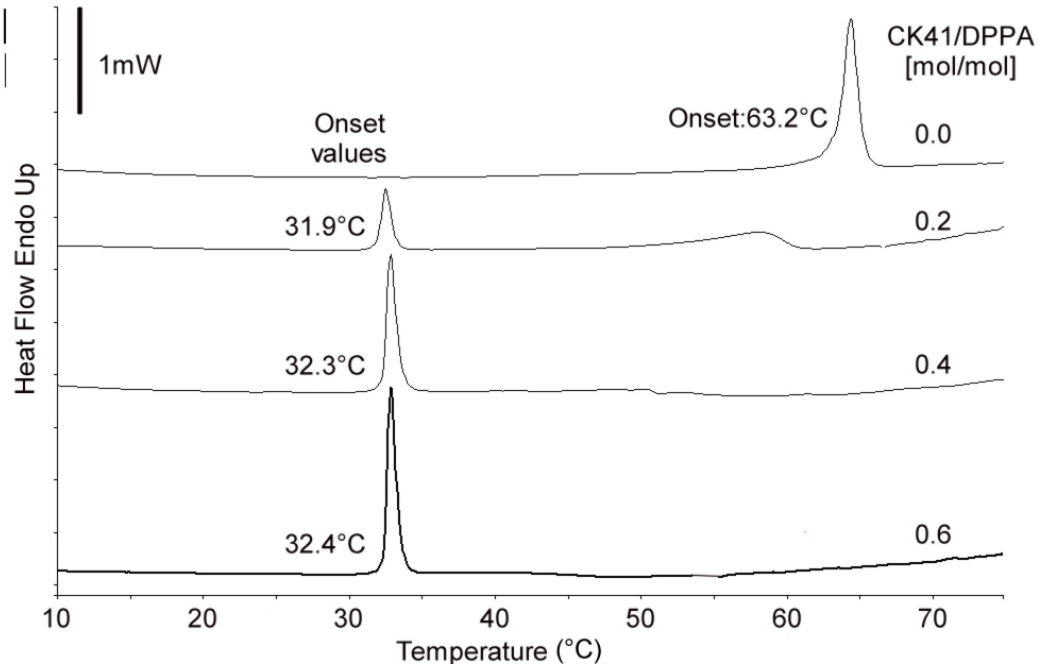


Figure 3-17: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance CK41 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, the divisions represent 0.5mW. ***Abscissa***: sample temperature in °C.

Experiments conducted					
1		2		3	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.05	31.4	0.2	31.3	0.5	31.2
0.1	33.3	0.4	30.9	0.8	30.8
0.2	33.8	0.6	30.8		
0.4	34.1				
0.6	34.1				
1.0	33.7				
Average ΔT_t	33.4 ± 0.42	Average ΔT_t	31.0 ± 0.15	Average ΔT_t	31.0 ± 0.20

Table 3-15: DPPA phase transition temperature reduction by CK41. The average value of ΔT_t from all the measurements (n = 11) amounted to 32.3 ± 0.44°C (mean value ± SEM).

3.2.2.1.15 KH241 (N,N-dimethyl-3-biphenylyl-3-phenylpropylamine)

This compound is a derivative of KH204 containing an additional phenyl moiety in the propyl chain.

The results of a representative experiment carried out with this compound, having a log P value of 3.36*, are shown in the figure below. Here, a detergent effect of the substance was observed at a molar ratio between 0.5 and 1.0.

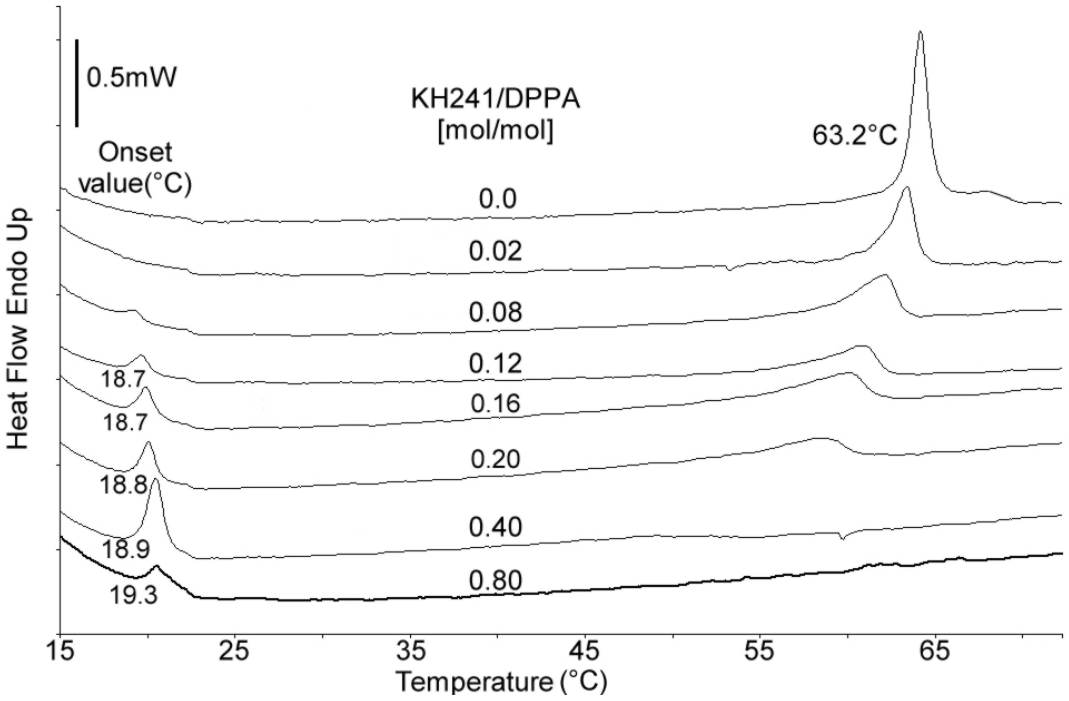


Figure 3-18: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance KH241 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 0.5mW. ***Abscissa***: sample temperature in °C.

Experiments conducted			
1		2	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.12	44.5	0.2	42.5
0.16	44.5	0.4	43.1
0.20	44.4	0.6	42.3
0.40	44.3		
0.80	43.9		
Average ΔT_t	44.3 ± 0.11	Average ΔT_t	42.6 ± 0.24

Table 3-16: DPPA phase transition temperature reduction by KH241. The average value of ΔT_t from all the measurements (n = 8) amounted to 43.7 ± 0.33°C (mean value ± SEM).

Summary table

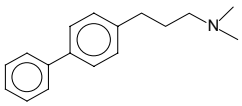
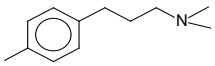
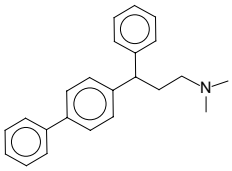
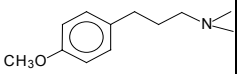
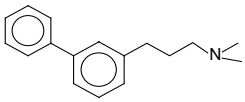
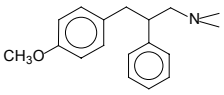
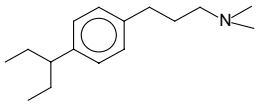
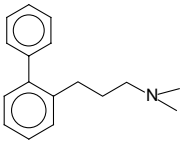
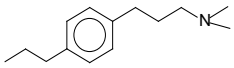
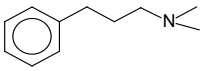
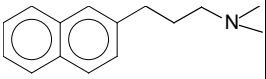
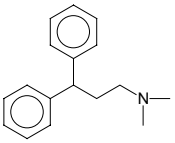
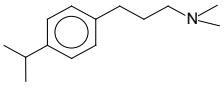
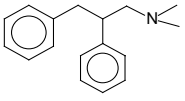
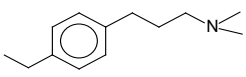
Test substance	ΔT_t (°C)	SEM (°C)	n	log P	Test substance	ΔT_t (°C)	SEM (°C)	n	log P
KH204 	53.1	0.16	7	3.16	KH211 	32.8	0.26	6	2.00
KH241 	43.7	0.33	8	3.36*	KH216 	32.8	0.13	10	1.48
CK92 	41.1	0.26	7	3.03	CK41 	32.3	0.44	11	n.d.
CK19 	40.9	0.04	7	3.68	CK53 	29.8	0.22	7	2.72
KH213 	40.9	0.25	7	2.89	KH210 	28.6	0.10	8	1.50
KH220 	39.9	0.22	10	2.62*	CK94 	27.5	0.09	6	2.35
KH214 	36.8	0.33	6	2.75	CK84 	27.1	0.07	5	2.27
KH212 	35.7	0.20	6	2.45					

Table 3-17: Reduction of the phase transition temperature of DPPA liposomes, ΔT_t by the phenylpropylamine derivatives in order of ΔT_t reduction beginning with the most active substance. The temperatures used were the onset temperatures. n represents the number of measurements considered in determining the mean ΔT_t. SEM: Standard error of the mean. The log P: logarithm of the octanol/buffer partition coefficient. The values were determined by C. Klein using reverse-phased HPLC. Those marked with an asterisk were measured by M. Klingmüller.

3.2.3 Muscarinic allosteric modulators

3.2.3.1 W84

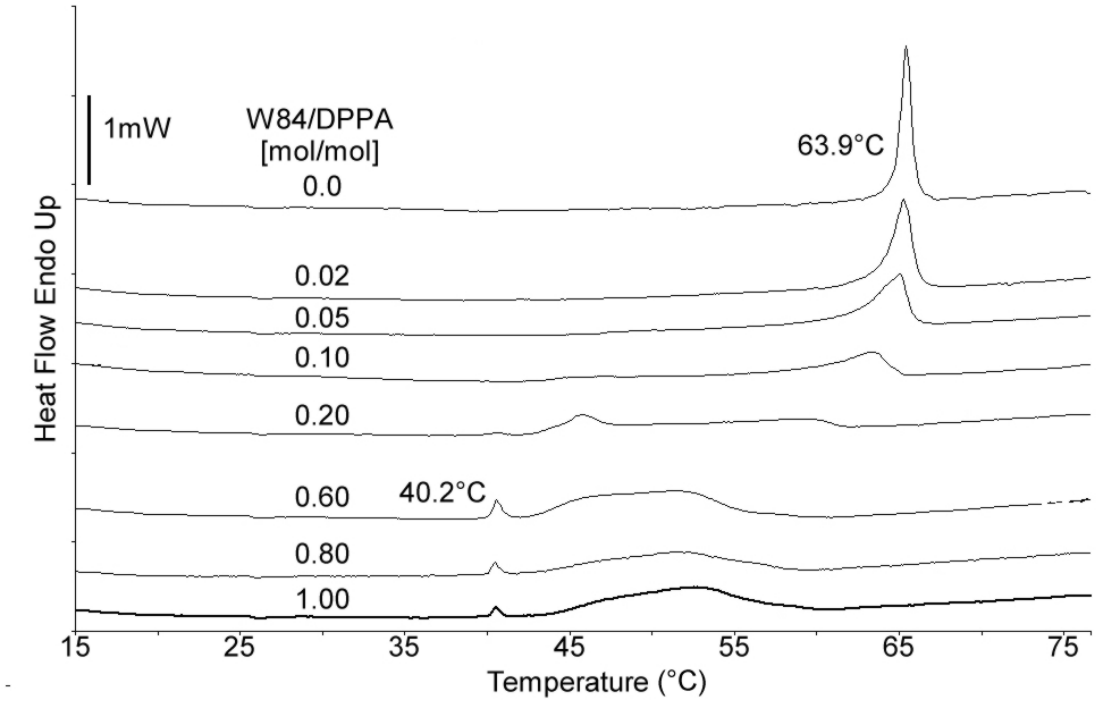


Figure 3-19: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance W84 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C. Representative experiment from a total of 3 experiments.

A very broad drug-induced signal is preceded by a relatively small peak, with the latter appearing at 40°C. The table below summarises the results obtained from the measurements carried out with W84 in DPPA liposome dispersions.

Experiments conducted					
1		2		3	
Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)	Molar ratio	ΔT_t (°C)
0.4	23.8	0.6	23.7	0.6	23.8
0.6	23.8	0.8	24.1	0.8	24.0
0.8	23.9	1.0	23.8		
1.0	23.6				
1.2	23.7				
Average ΔT_t	23.8 ± 0.05	Average ΔT_t	23.9 ± 0.12	Average ΔT_t	23.9 ± 0.10

Table 3-18: DPPA phase transition temperature reduction by W84. The average value of ΔT_t from all the measurements (n = 10) amounted to 23.8 ± 0.05°C (mean value ± SEM).

3.2.3.2 Silicon containing W84 derivative TD5

The silicon containing W84 derivative elicits a different allosteric behaviour from that of W84 as observed in radioligand binding studies (Duda-Johner, 2002). Several experiments were performed using TD5 from different batches to confirm the signal recorded at a mole fraction of test substance to phospholipid of 1.0. The resulting signals were similar.

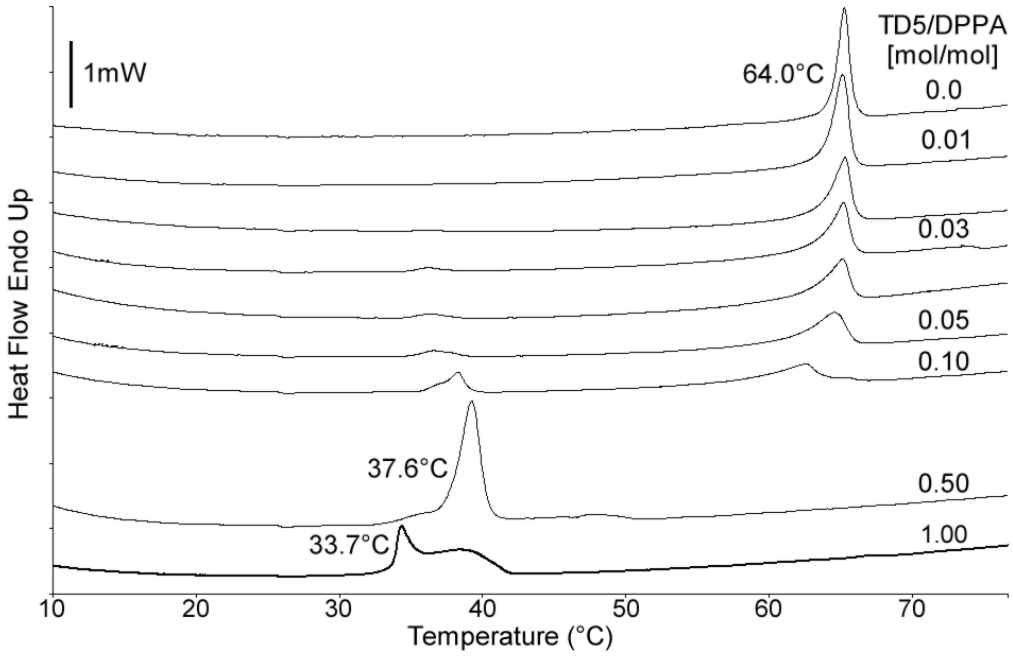


Figure 3-20: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance TD5 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

Here, the main test substance-induced transition signal that appears at a molar ratio 0.5 gets broader and a further peak emerges with a T_t of 33.7°C, an indication of a possible superimposition of a sharp and a broad component.

3.2.3.3 Duo3

The structure of this bispyridinium compound is shown in the materials section. The results of experiments with this substance in DPPC liposomes are presented in the next figure.

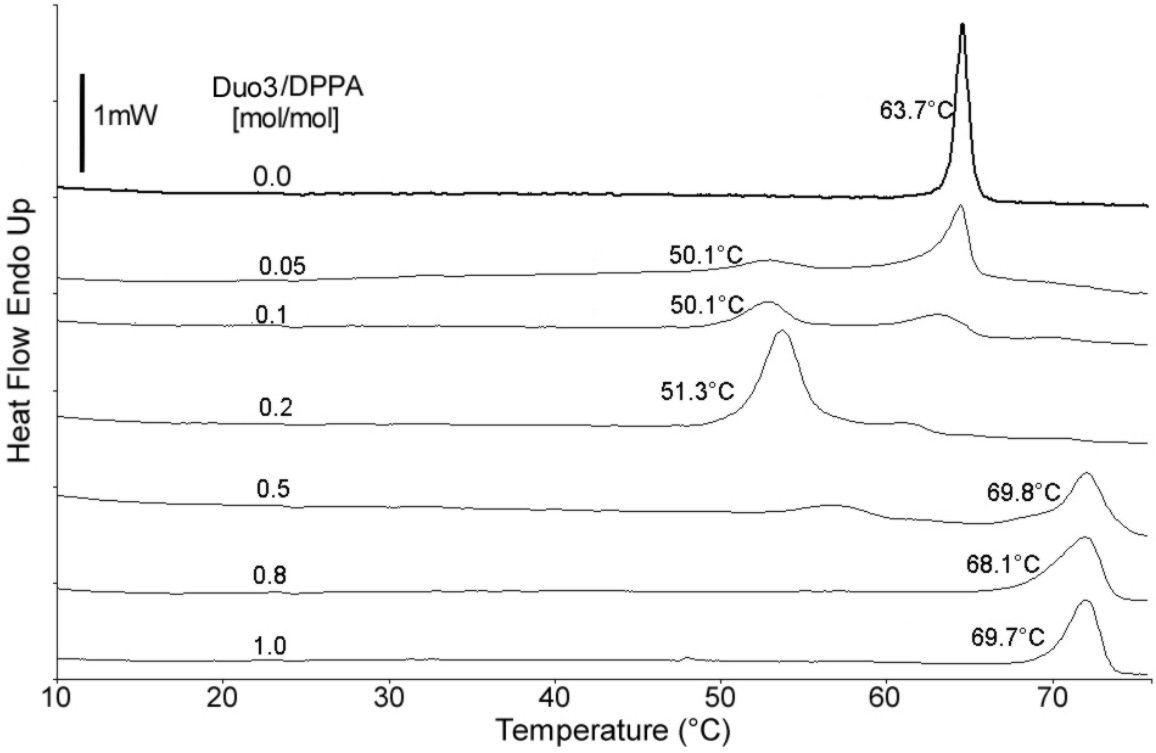


Figure 3-21: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance Duo3 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

It is worth noticing the initial reduction in T_t and the eventual increase at higher molar ratios. The average measured reduction in T_t was 13.4 ± 0.37°C (mean ± SEM) while the average increase amounted to 6.2 ± 0.30°C (mean ± SEM). The average reduction value was approximately twice the increase value.

3.2.3.4 WDuo3

The behaviour of WDuo3 varies slightly from that of Duo3. Multiple peaks appear at mole fractions from 0.05 to slightly over 0.2 at temperatures near 41°C. Eventually, peaks emerge at a temperature higher than that of pure DPPA, but more spiky and at a lower temperature than those of Duo3.

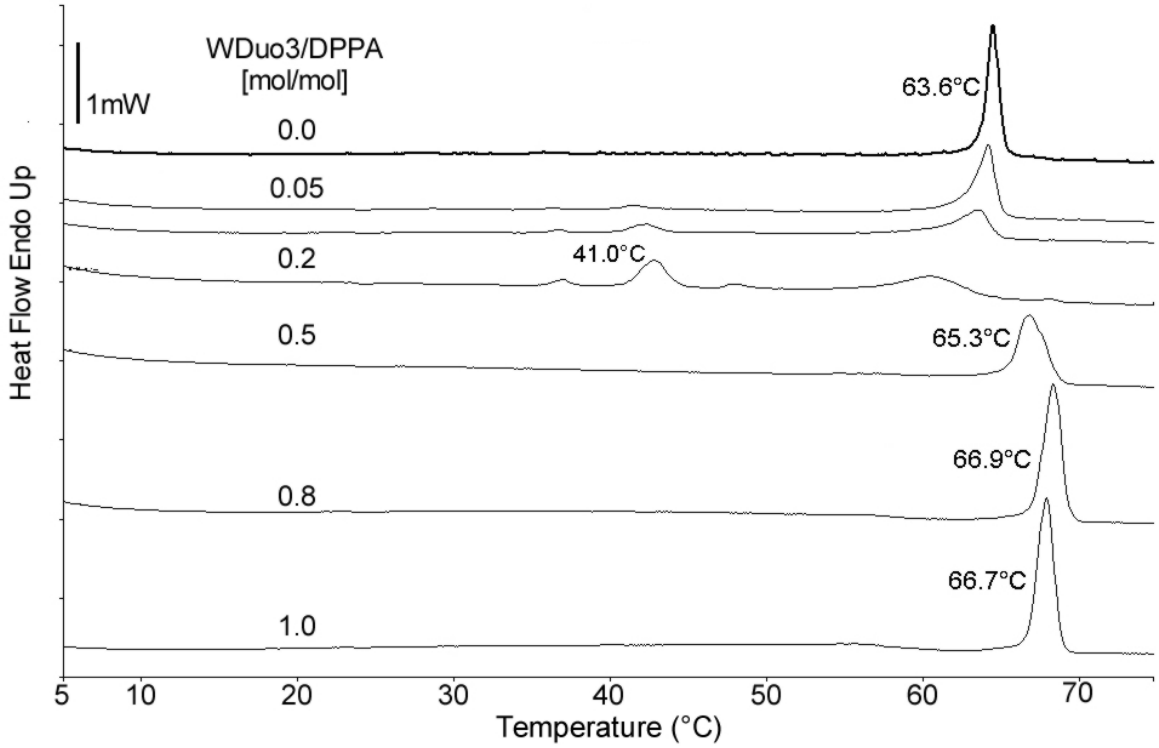


Figure 3-22: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance WDuo3 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

The average increase in T_t amounted to 2.4 ± 0.36°C (mean ± SEM; n = 6). This value is much smaller than that obtained from Duo3.

3.2.3.5 Naphmethonium (MM3A)

The compound naphmethonium (section 2.3.3.1 for structural formula) is a further derivative of the substance W84 that was investigated. One of the phthalimidopropyl residues in W84 is replaced by the more voluminous naphthylimido-ß-dimethylpropyl residue. The multi-peak signals indicate interactions that differ from those that take place between W84 and the phospholipids. T_t is finally reduced to a value slightly less than 40°C.

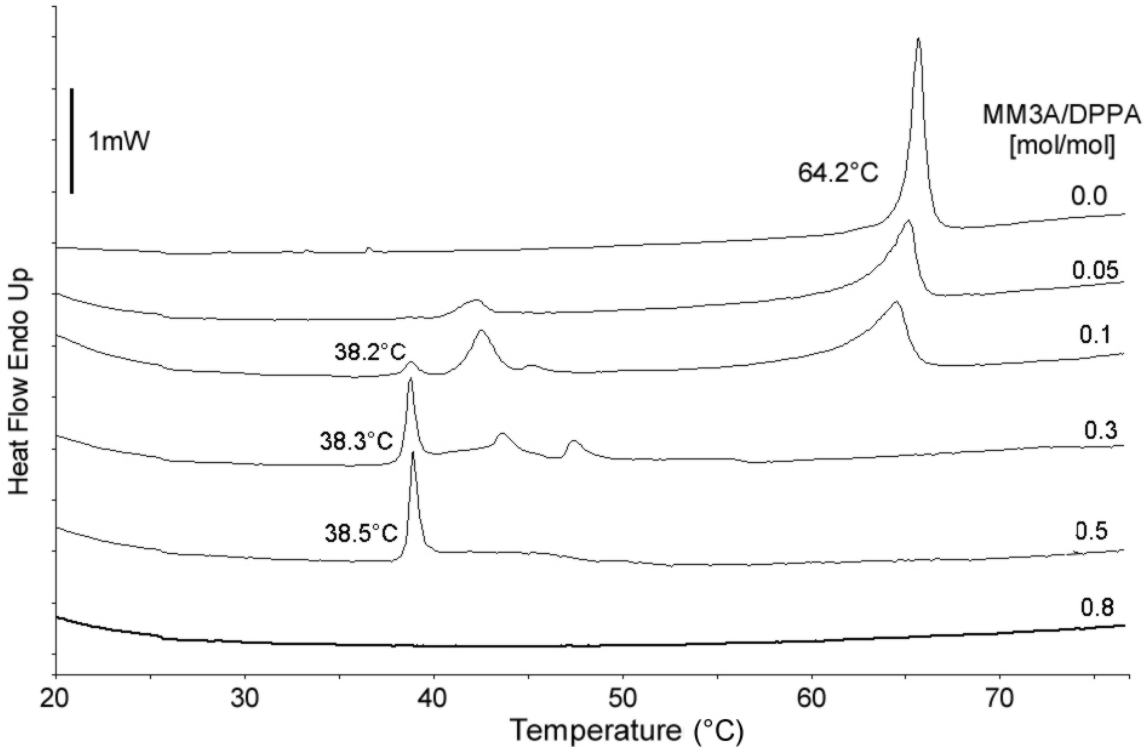


Figure 3-23: Original thermograms obtained from DSC measurements, using DPPA-liposome dispersions containing the substance naphmethonium in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 0.5mW. ***Abscissa***: sample temperature in °C.

A multi-peak signal eventually becomes a single peak signal, with the average ΔT_t value of the peaks with the lowest onset temperatures being 26.0 ± 0.16°C (mean ± SEM; n = 6).

3.3 Test of reproducibility of measurements

It is of pivotal importance, that the values obtained from the various experiments be reproducible to ensure reliability and validity of the results. Tests were therefore conducted with the same sample and the same substance measured several times in succession. These measurements were carried out using liposome suspensions from pure DPPA, as well as liposome suspensions containing the substance TD5 in two mole proportions. The results of two of these experiments are shown below.

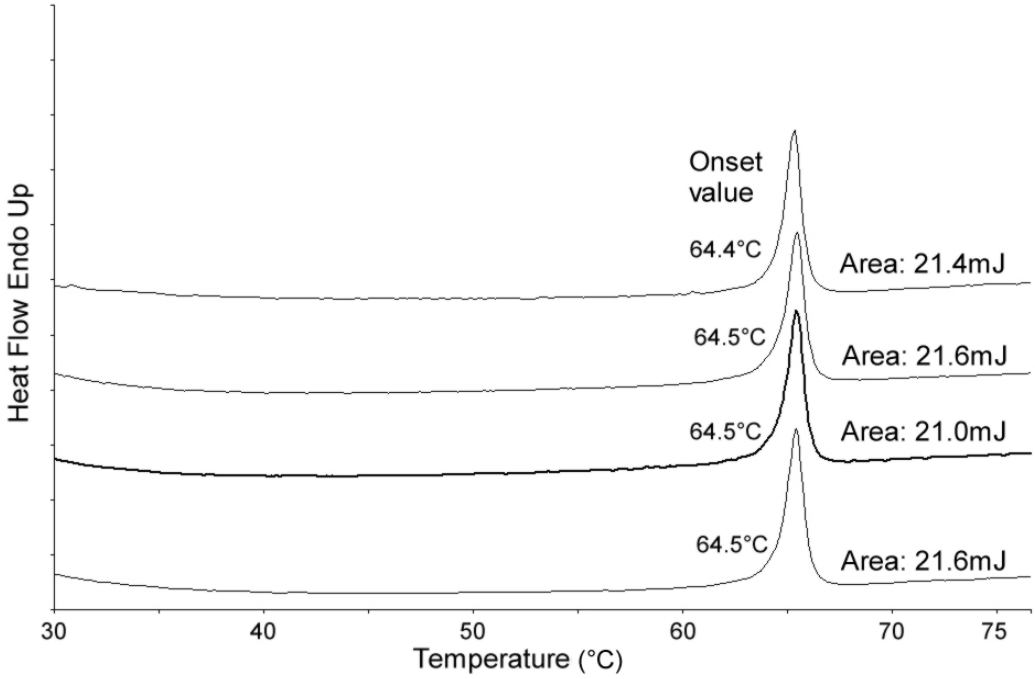


Figure 3-24: Thermograms obtained from one of the experiments carried out using pure DPPA liposomes to test the reproducibility of the results obtained with the apparatus. Also noticeable is the absence of a pre-transition peak. Further experiments carried out with different samples are not shown graphically, but the results from these are summarised in table 3-19. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

Experiments conducted with pure DPPA liposomes			
	Sample 1	Sample 2	Sample 3
	T_t (°C)	T_t (°C)	T_t (°C)
1	65.1	64.8	64.7
2	65.2	65.0	64.7
3	64.9	64.7	64.7
4	64.9	64.8	64.8
Average T_t (°C)	65.0 ± 0.08	64.8 ± 0.06	64.7 ± 0.03

Table 3-19: DPPA phase transition temperatures obtained using different samples of DPPA liposomes. Given above are the average values obtained per sample ± standard error of the mean. The average value of T_t from all the measurements (n = 12) amounted to 64.9 ± 0.17°C (mean value ± SEM).

In the previous case, measurements were carried out repeatedly in close succession. In the next case, the substance TD5 was also used. The results obtained are shown below.

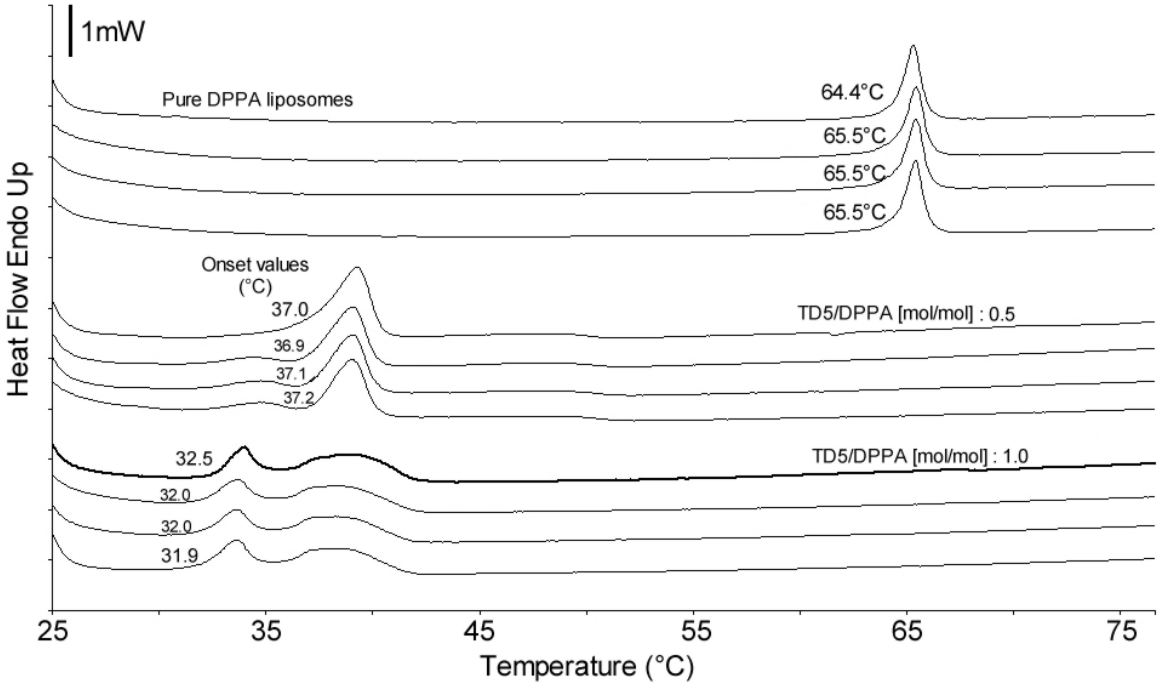


Figure 3-25: Original thermograms obtained from DSC measurements, using the same samples of pure DPPA-liposome dispersions and DPPA liposome dispersions containing the substance TD5 in a substance to phospholipid ratio of 0.5 and 1.0, respectively.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

In both cases, the results are explicit enough and it would not be expected that the results obtained from the other experiments would be different. Statistical tests of various values resulting from experiments like the two above showed no statistical difference in the values and a very small standard deviation. In the cases considered in these statistical analyses, the degrees of freedom were generally higher.

3.4 Test to rule out a possible influence of the solvent dimethylsulfoxide (DMSO)

DMSO was the least volatile of the solvents used in dissolving test compounds that were eventually used in preparing the liposomes investigated in this work. It was therefore deemed necessary to perform tests with a substance that needed to be dissolved in DMSO as solvent and compare them with the results of tests performed with liposomes where the test substances were soluble in buffer solution and were hence dissolved directly in buffer. After measuring the required quantity of substance

in the DMSO solution, the solvent was then evaporated using the vacuum pump dryer, Speed Vac® (section 2.5.1). The results from one such experiment are shown in figure 3-26.

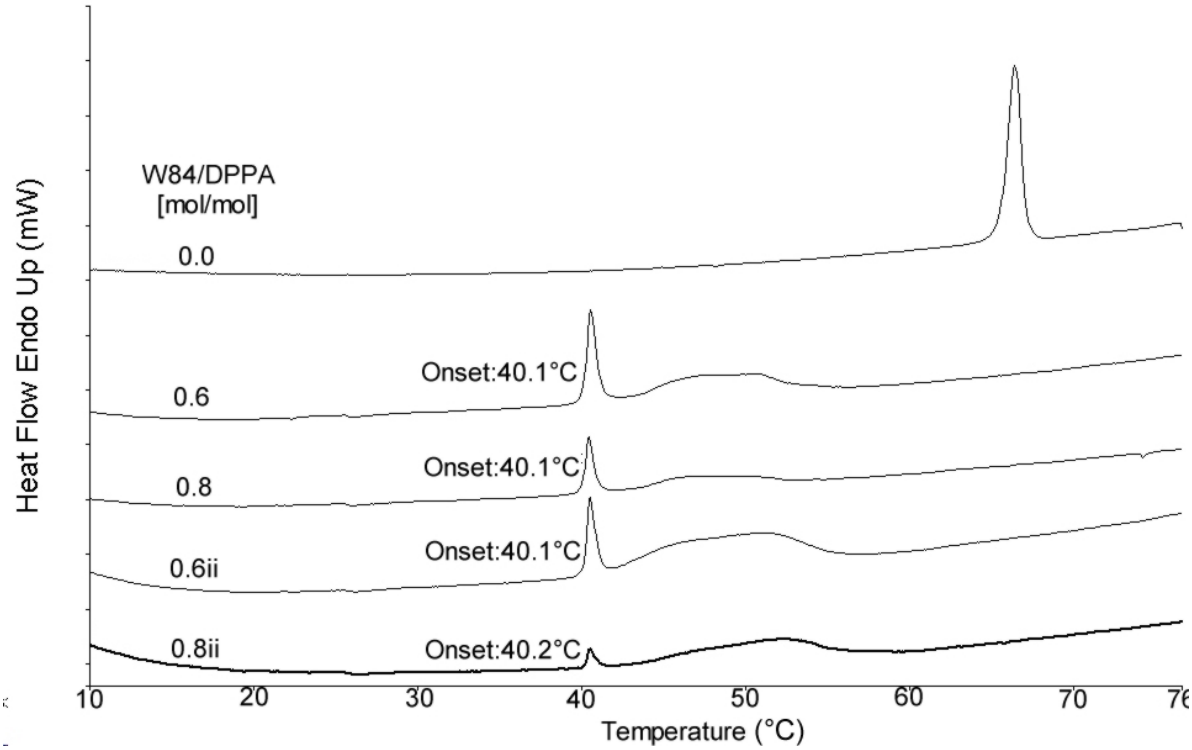


Figure 3-26: Thermograms obtained from experiments carried out using pure DPPA liposomes and liposomes containing the substance W84 in two concentrations to test the possible influence of the solvent used in the experiments performed. The thermograms marked with (ii) resulted from experiments in which DMSO was used as solvent. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

The results show that the onset temperatures are unaffected by the use of the solvent DMSO. This is an indication that the solvent is evaporated to dryness before the residue is resuspended in buffer solution.

3.5 Results from experiments conducted with DPPC liposomes

Experiments using DPPC as phospholipid in the liposome dispersion resulted in thermograms that differed from those obtained using DPPA. This is not surprising, considering that the head groups of both phospholipids differ not only in size but also in charge density. Whereas the reduction in phase transition temperature was usually a constant value using DPPA, with DPPC a gradual reduction in T_t with increasing molar ratio of test substance to phospholipid was usually observed. Also, the reduction of the onset temperature brought about by the test compounds with DPPC did not generally attain the absolute value brought about by these substances with DPPA in the corresponding molar ratios.

3.5.1 Propranolol

Experiments were carried out using propranolol in liposome dispersions with DPPC and the resulting original thermograms from such experiments are shown in the following three figures in different molar ranges.

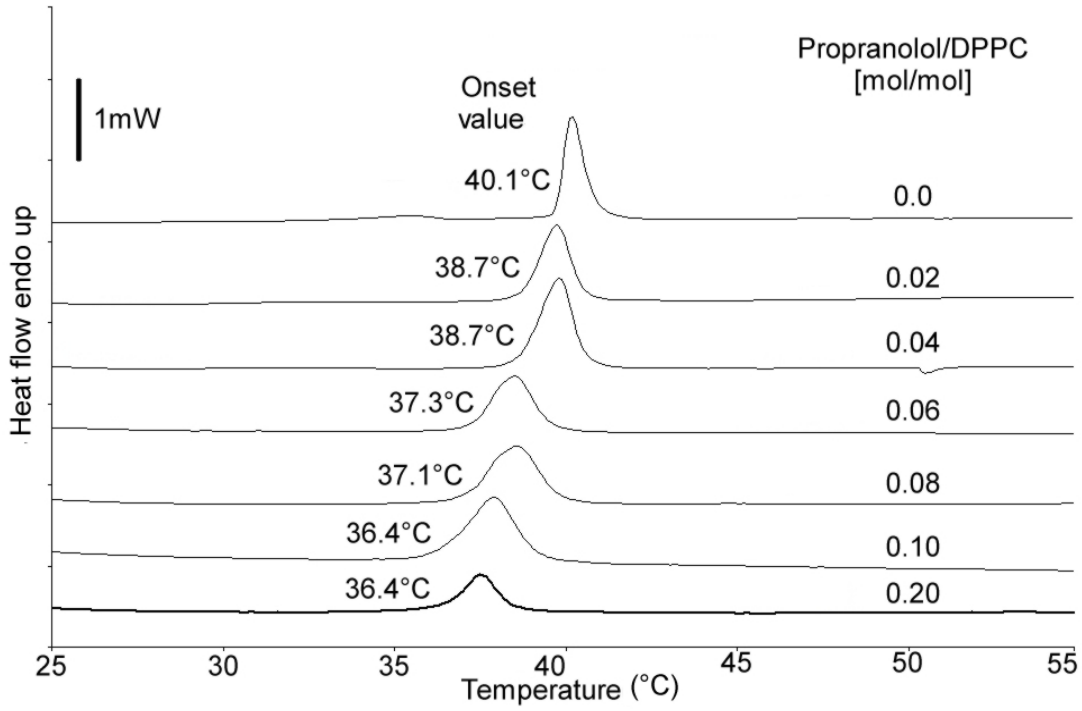


Figure 3-27: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance propranolol in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

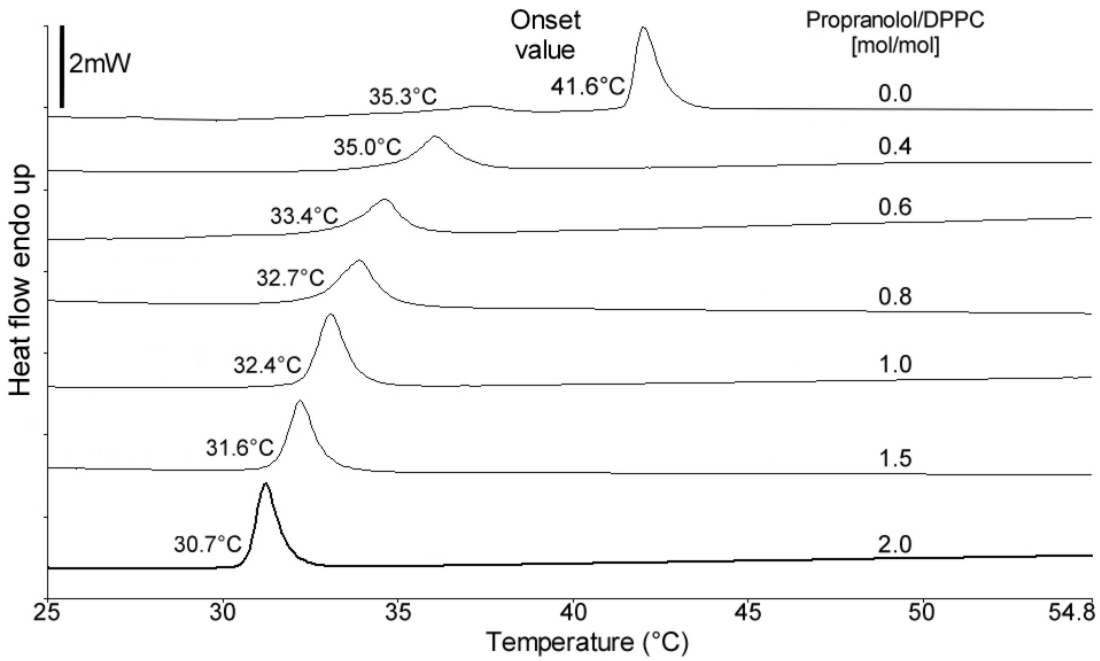


Figure 3-28: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance propranolol in the indicated molar ratios. ***Ordinate***: endothermic heat flow. As shown, each division represents 2mW. ***Abscissa***: sample temperature in °C.

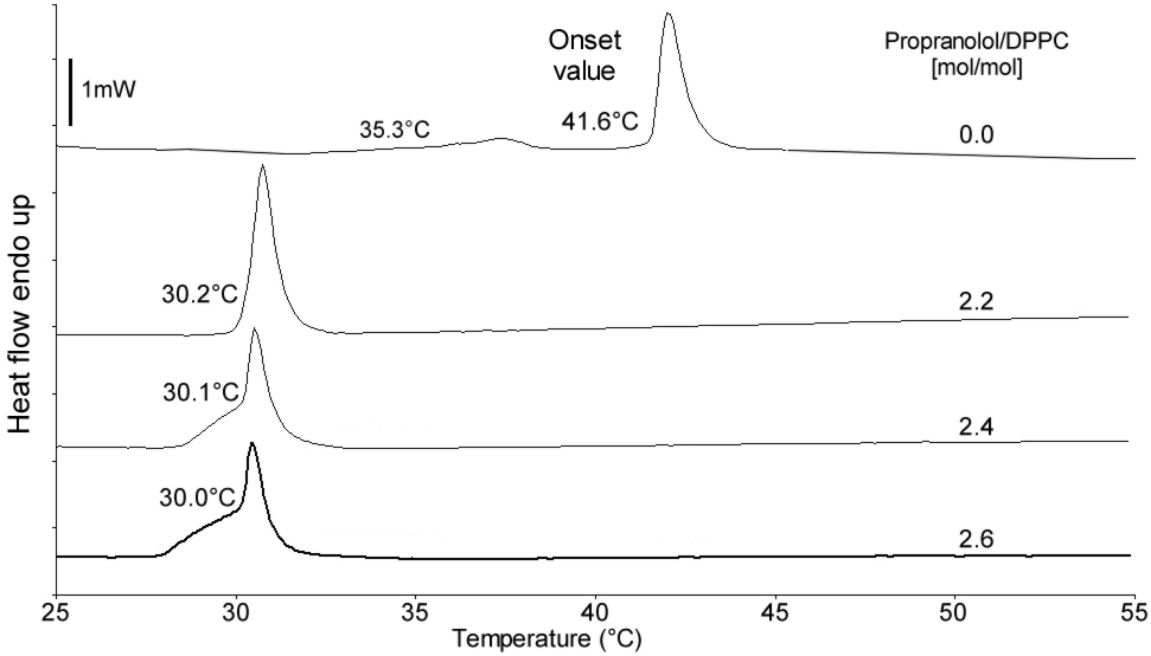


Figure 3-29: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing propranolol in the indicated molar ratios, the highest that were measured.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

The graphs show thermograms resulting from the use of increasing molar ratios. The first experiment and the last two were performed on separate days, respectively.

As observed in other studies with cationic amphiphilic compounds (Kursch et al., 1983; Hanpft and Mohr, 1985; Hanpft, 1987; Mohr and Struve, 1991), a gradual reduction in the onset value is evident here, with the pre-transition signal eventually disappearing completely. The shape of the signal also changes with increasing molar ratio. The signal gets broader over a certain range of molar ratios and finally becomes narrower again.

The dose-effect curve below summarizes the results obtained from the experiments above. The dose-effect curves, including those of subsequent substances, were not fitted to suite any given experimental model. The fit determined the nature of the curve, and all the curves were not based on any specific experimental equation template. Using algorithms based on built-in equations, the program prism computed the data and determined the best fit from among a number of equations.

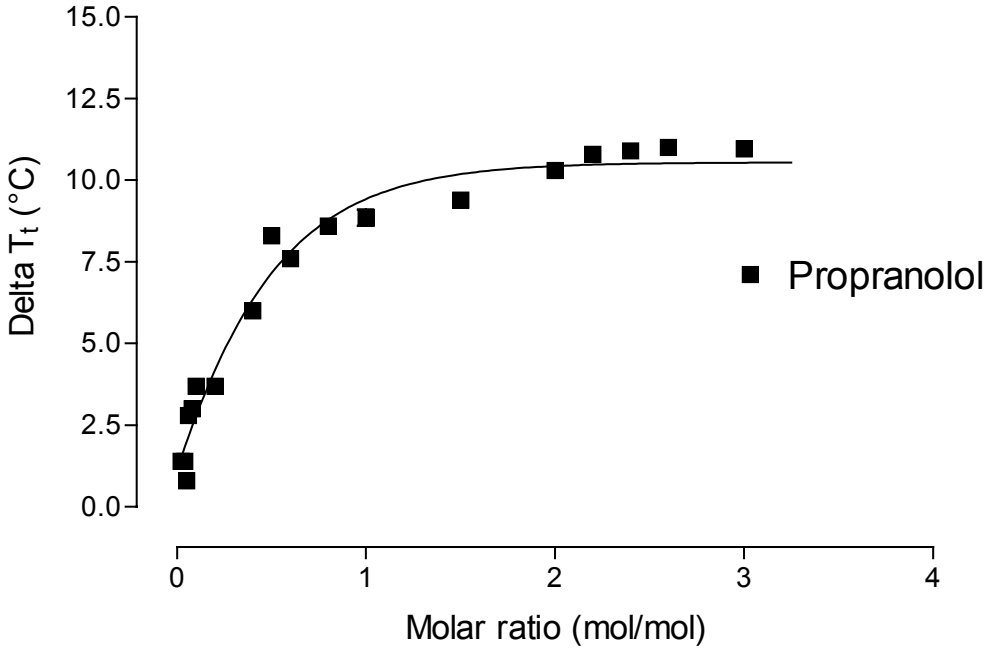


Figure 3-30: Dose-effect curve illustrating the influence of propranolol on the phase transition temperature of DPPC liposomes.
***Ordinate**:* Reduction of the phase transition temperature. ***Abscissa**:* molar ratio of the test substance to DPPC.

The plateau lies at a ΔT_t of about 11°C. The curve is generated from the results of two experiments including those shown in the thermograms in the previous three figures.

3.5.2 Phenylpropylamines

Results of experiments conducted with various phenylpropylamines as test substances in DPPC liposomes are expressed in graphs showing the reduction of the phase transition temperature. These results are shown for the various substances in the next pages.

3.5.2.1 KH210

As already mentioned above, this was the parent compound and its structure is present in all the other investigated phenylpropylamines. Experiments with this compound in DPPC liposome dispersions yielded thermograms shown in the figure below.

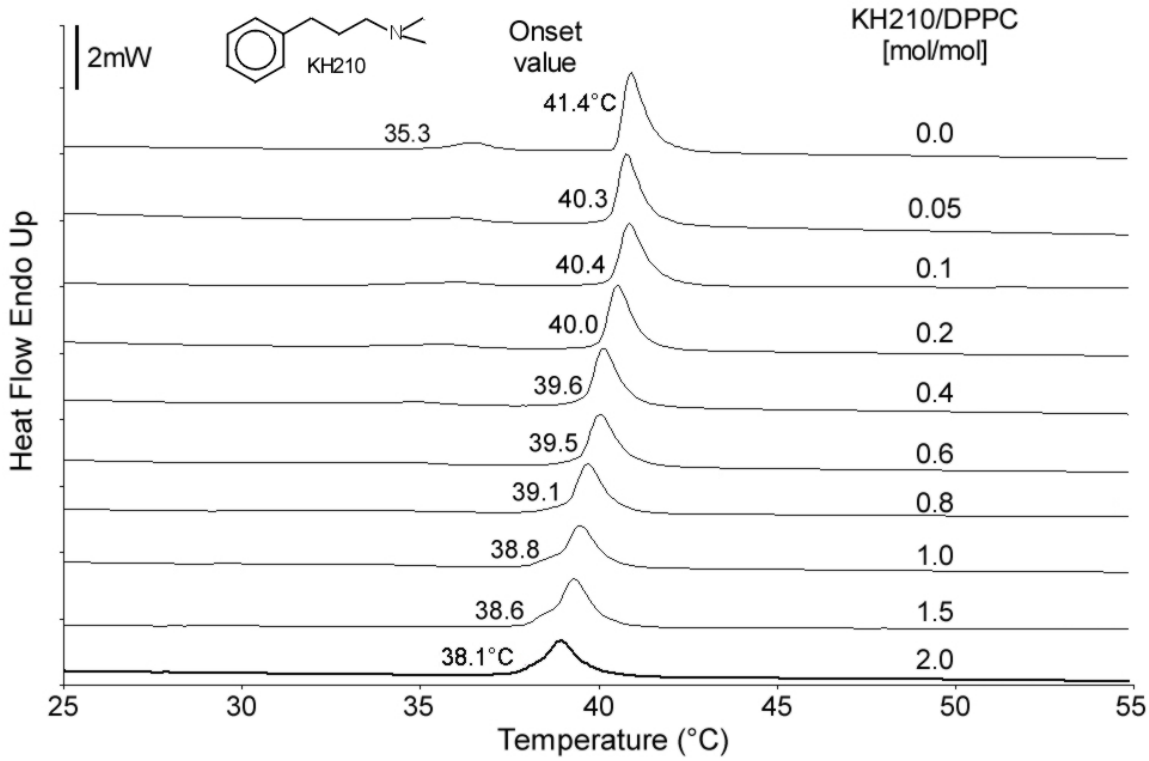


Figure 3-31: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH210 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 2mW. ***Abscissa***: sample temperature in °C.

There was no observable detergent effect up to the measured molar ratio of 3.5. At this molar ratio the peaks of the resulting thermograms, of which are not shown in the graph above, became narrower than the peak obtained from measuring the dispersion at the molar ratio of 2.0 shown above.

A dose effect curve of the obtained results is shown in the next figure.

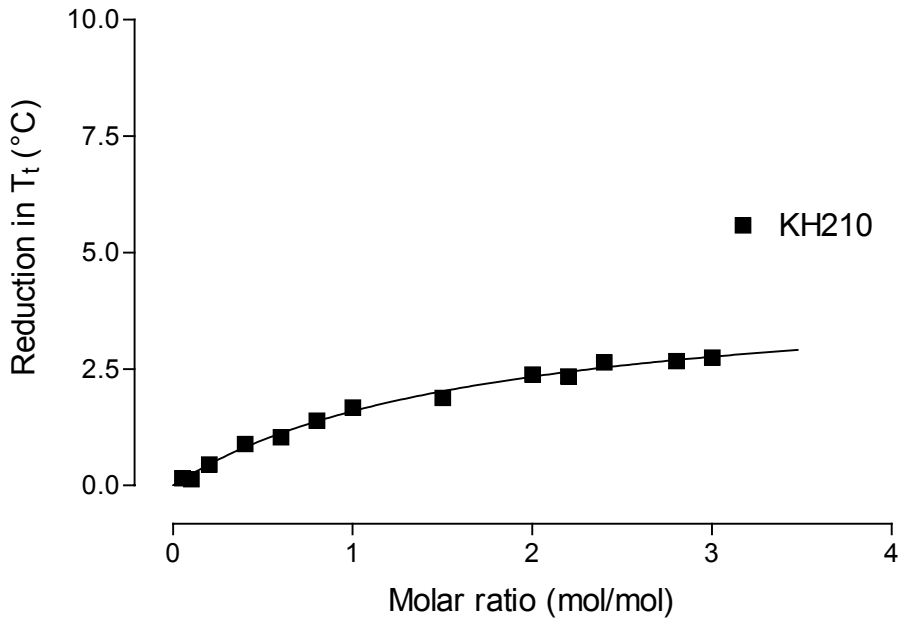


Figure 3-32: Dose-effect curve illustrating the influence of the phenylpropylamine KH210 on the phase transition temperature of DPPC liposomes.

***Ordinate**:* reduction of the phase transition temperature. ***Abscissa**:* molar ratio of the test substance to DPPC.

Shown here are the mean values from two experiments. A total of six values were determined in the second experiment, spread over the entire measured range shown above. The standard errors of the mean are also represented but are not apparent due to their small absolute values and the relative sizes of the symbols and the magnitude of the ordinate scale. The scale has a maximum value of 10°C to facilitate comparison with curves obtained using subsequent substances. The plateau is attained at a ΔT_t value of about 2.7°C. The detergent effect had not ensued at the maximum molar ratio measured and liposomes were still visible under the light microscope at this molar ratio.

3.5.2.2 KH211

The presence of the methyl group in *p*-position of the benzene ring causes a further depressing action in T_t compared to the parent compound (Fig. 3-32), as is evident from the figure below.

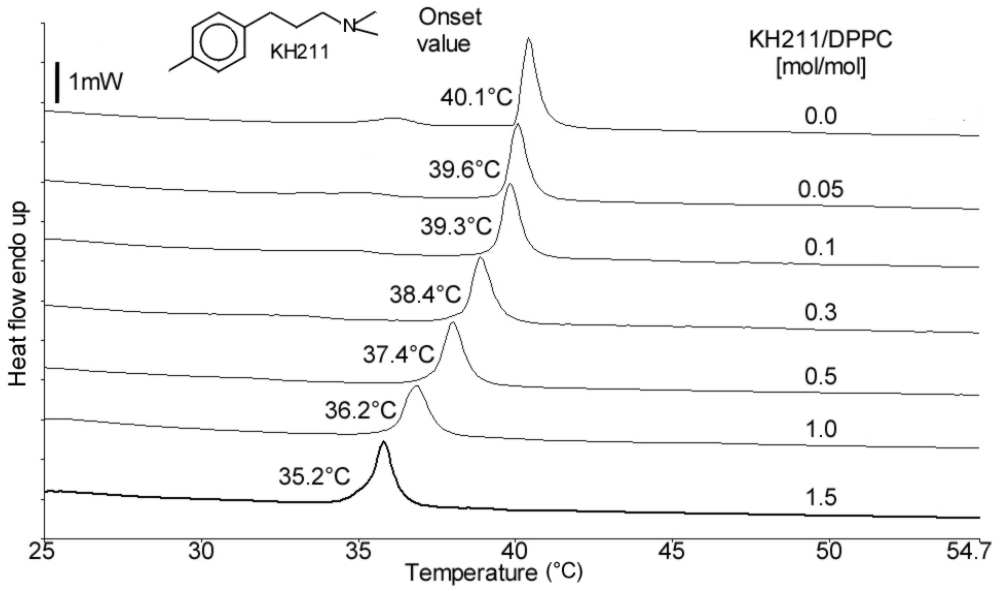


Figure 3-33: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH211 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

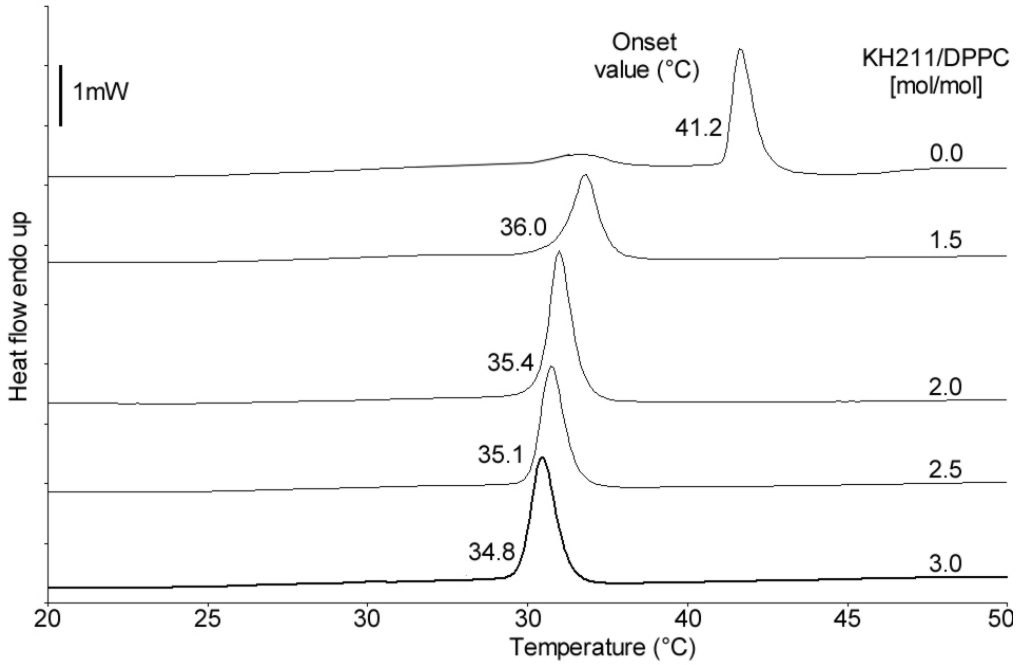


Figure 3-34: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH211 in the indicated high molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

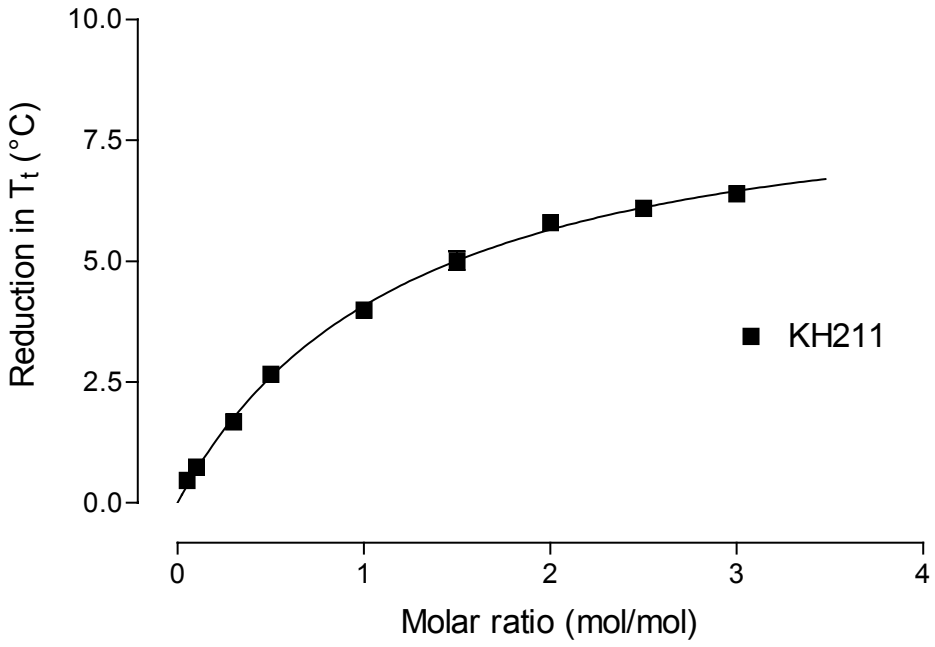


Figure 3-35: Dose-effect curve illustrating the influence of the phenylpropylamine KH211 on the phase transition temperature of DPPC liposomes.
***Ordinate**:* reduction of the phase transition temperature. ***Abscissa**:* molar ratio of the test substance to DPPC. Shown here are the mean values with the standard errors of the mean representing the error bars. The last three values resulted from the one experiment carried out to determine the maximum measurable molar ratio. The extrapolated curve produced a ΔT_t plateau value of about 6.5°C.

Like with many of the other phenylpropylamines with aliphatic substituents, a milky white dispersion was observed up to the measured molar ratio of 3.0 without the appearance of a detergent effect. As can be seen in figure 3-34, the curves maintain their integrity and a clear onset temperature could be determined.

3.5.2.3 KH216

The influence of this substance on the T_t of DPPC is similar to that brought about by the parent compound KH210. A detergent effect was not observed even at the maximum measured molar ratio of 3.0, and the peaks maintained their integrity even at this high molar ratio.

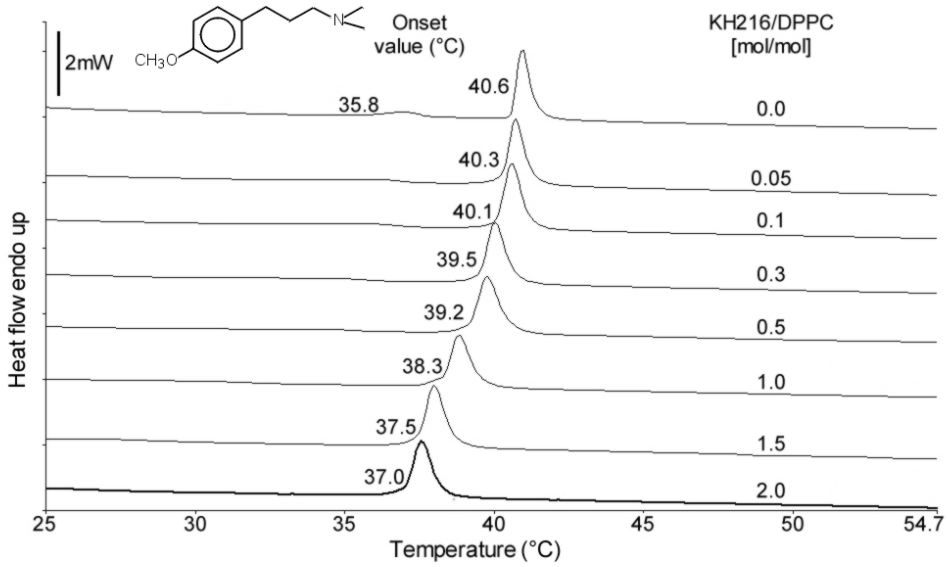


Figure 3-36: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH216 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 2mW. ***Abscissa***: sample temperature in °C. Shown here are thermograms from one of two experiments.

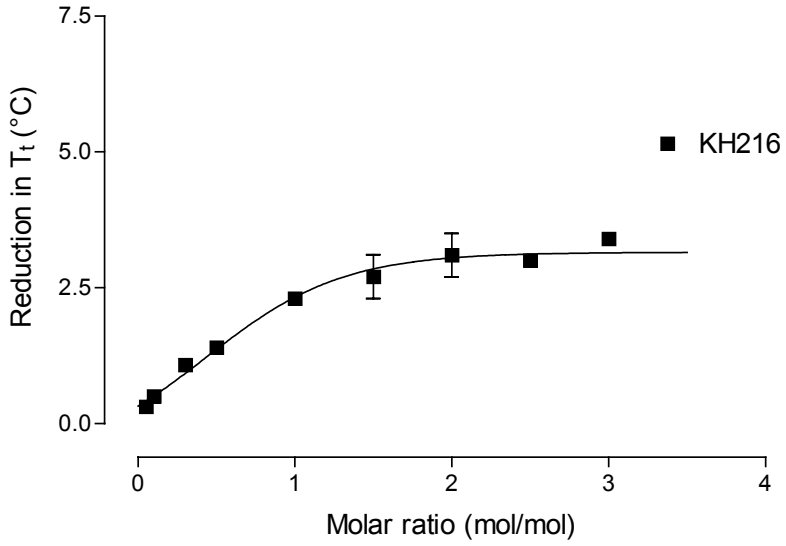


Figure 3-37: Dose-effect curve illustrating the influence of the phenylpropylamine KH216 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The last two values are from the one experiment carried out to determine the maximum measurable molar ratio. The extrapolated curve produced a ΔT_t plateau value of about 3°C.

3.5.2.4 KH212

The substance brings about an even greater reduction in T_t than KH211.

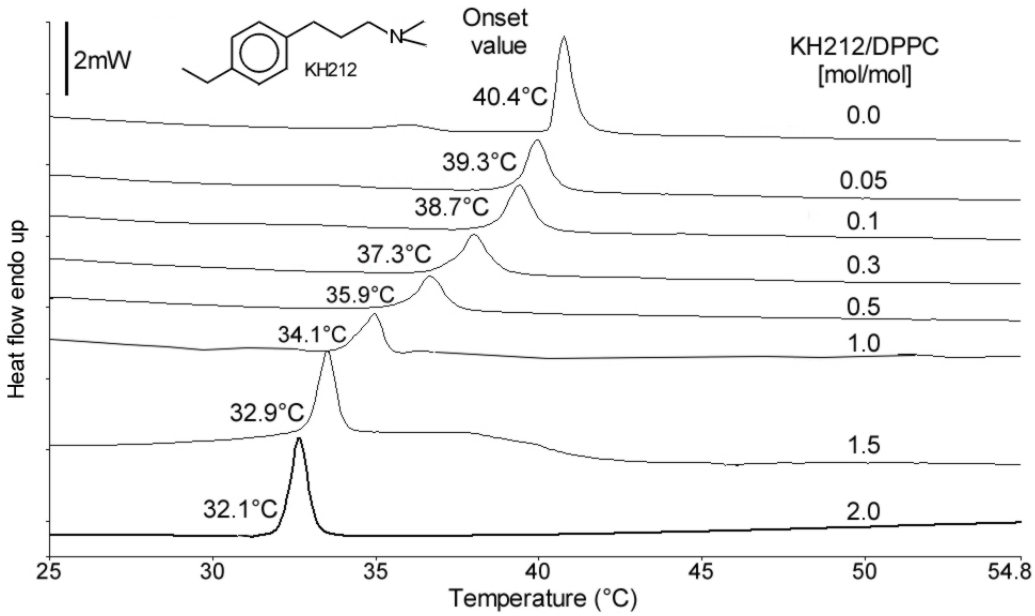


Figure 3-38: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH212 in the indicated molar ratios.
Ordinate: endothermic heat flow. ***Abscissa***: sample temperature in °C. Shown here are thermograms resulting from one of two experiments. Clearly defined thermograms were also achieved to the measured molar ratio of 3.0 with liposomes visible in the dispersions to the last molar ratio.

Here too, clearly defined thermograms were achieved to the molar ratio of 3.0 without the detergent effect ensuing.

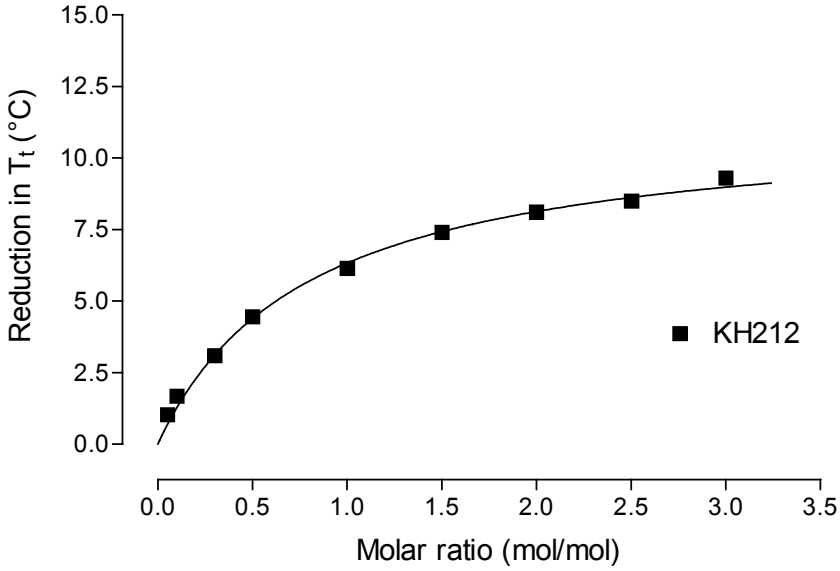


Figure 3-39: Dose-effect curve illustrating the influence of the phenylpropylamine KH212 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The extrapolated curve produced a ΔT_t plateau value of about 9°C.

3.5.2.5 KH214

Results from experiments carried out using this substance with a branched (isopropyl-) side chain are shown below.

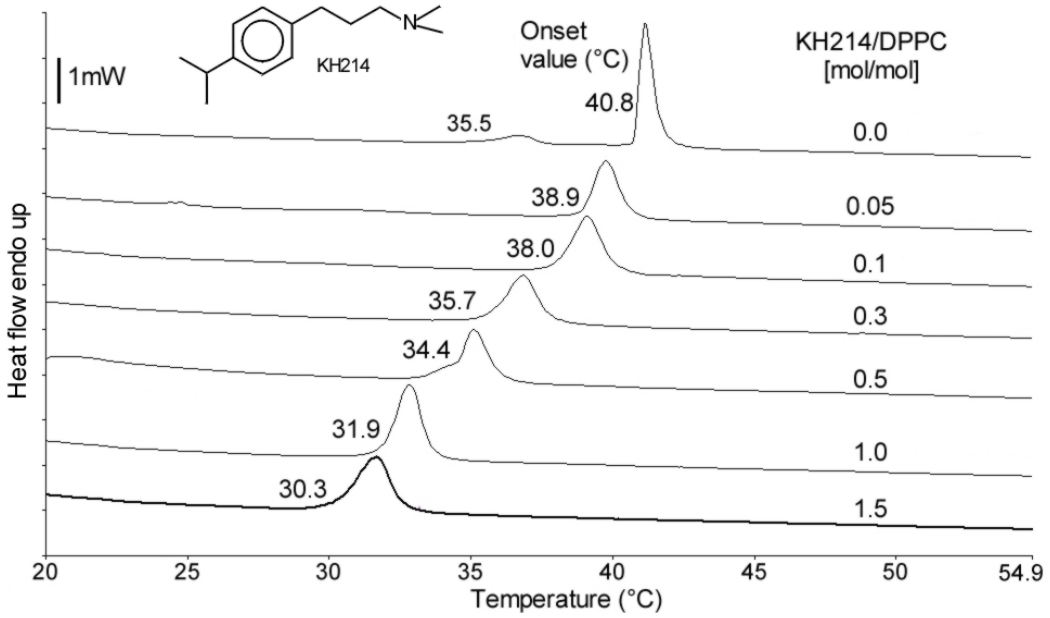


Figure 3-40: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH214 in the indicated molar ratios.
Ordinate: endothermic heat flow. ***Abscissa***: sample temperature in °C. Shown here are thermograms resulting from one of two experiments. At a molar ratio of 2.5, the dispersion became translucent when hot and cloudy at ambient temperatures. At a ratio 3.0, it became a clear solution.

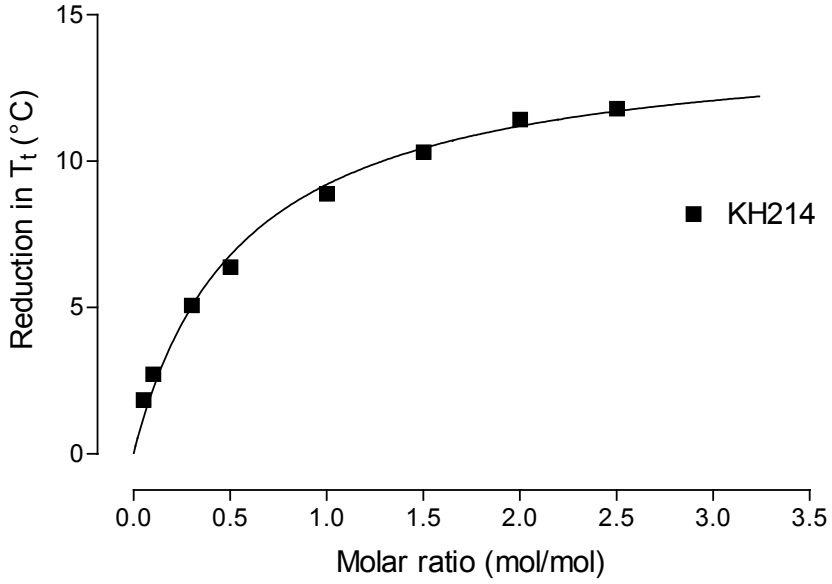


Figure 3-41: Dose-effect curve illustrating the influence of the phenylpropylamine KH214 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The scale goes beyond 10 units to accommodate the high ΔTt. The extrapolated curve produced a ΔT_t plateau value of about 12.5°C.

3.5.2.6 KH213

Here, the shapes of the peaks change after a molar ratio of 0.6 with the peaks becoming more symmetrical as shown below.

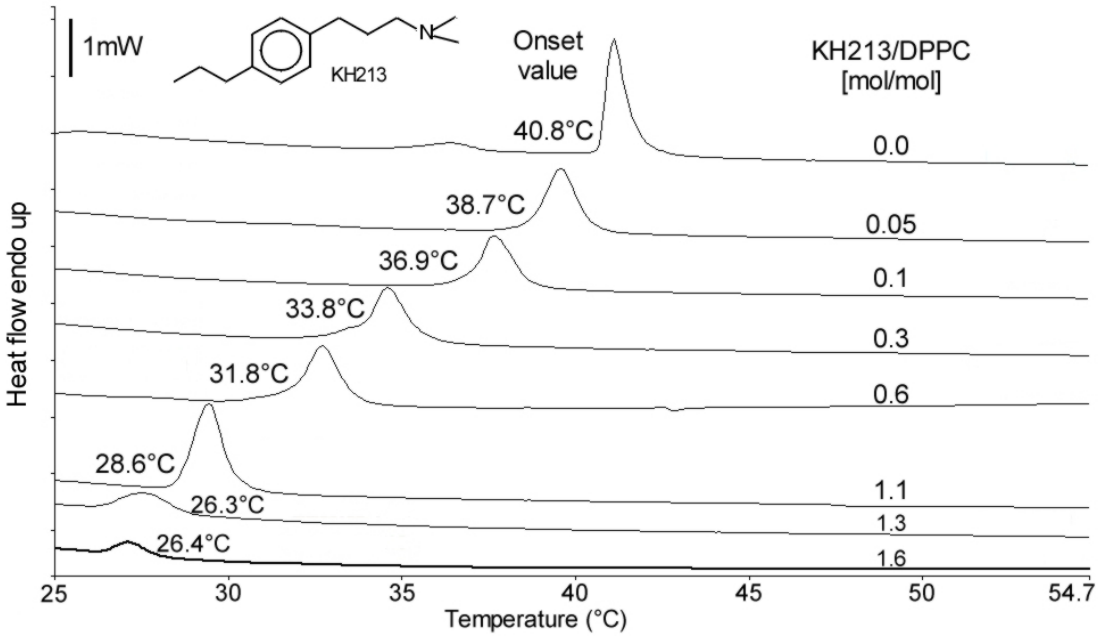


Figure 3-42: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH213 in the indicated molar ratios. Molar ratios above 1.6 did not produce any observable signals.
Ordinate: endothermic heat flow. ***Abscissa***: sample temperature in °C. Shown here are thermograms resulting from one of two experiments. The dispersions became very translucent at a molar ratio of 1.3 and clear at 1.6 and beyond that ratio. Measuring the exact T_t became difficult at these molar ratios.

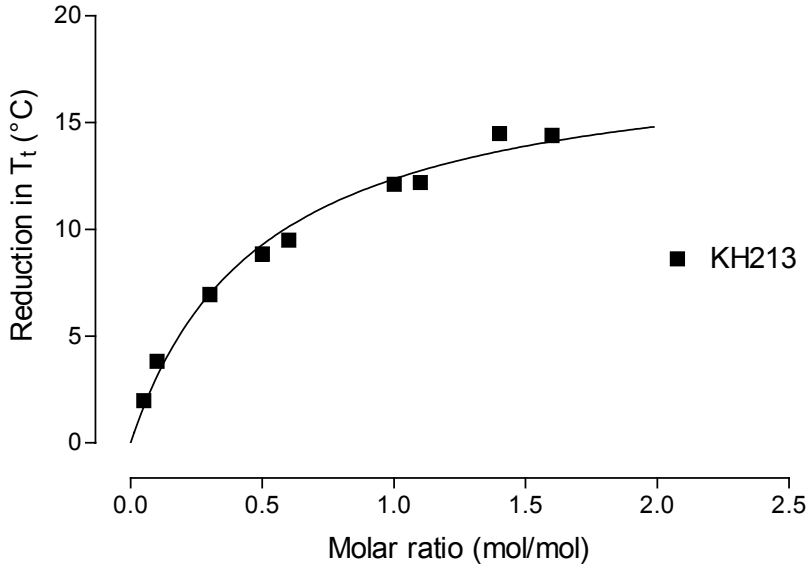


Figure 3-43: Dose-effect curve illustrating the influence of the phenylpropylamine KH213 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The extrapolated curve produced a ΔT_t plateau value slightly about 15°C.

3.5.2.7 CK19

The substance has a strong depressant effect on T_t as shown below although the broad base of the peaks and the manner in which the peaks deviated from the baseline made determining the exact T_t in some molar ratios difficult.

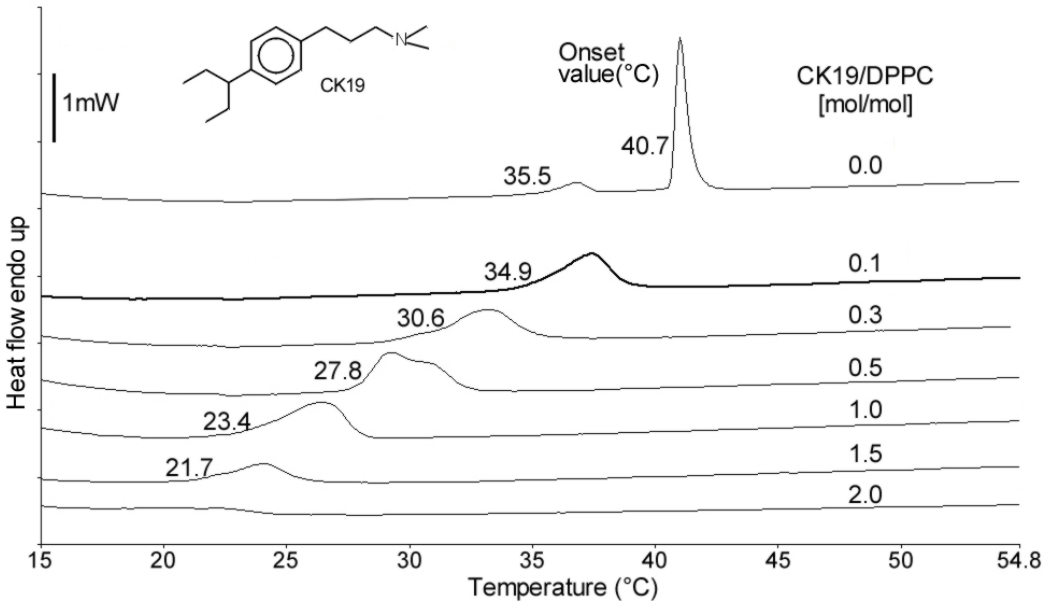


Figure 3-44: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance CK19 in the indicated molar ratios.
Ordinate: endothermic heat flow. ***Abscissa***: sample temperature in °C. Shown here are thermograms resulting from one of two experiments. The dispersions got from milky white and fluid through a viscose, cloudy stage and finally to a fluid colourless solution, indicating the detergent effect at a molar ratio of 2.0.

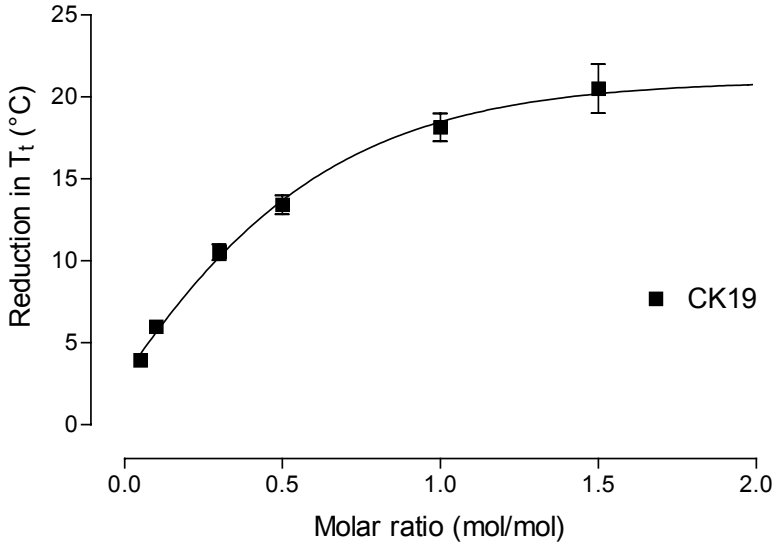


Figure 3-45: Dose-effect curve illustrating the influence of the phenylpropylamine CK19 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The extrapolated curve produced a ΔT_t plateau value slightly over 20°C.

3.5.2.8 KH220

The naphthalene derivative brought about a transition temperature change shown in the graph below. The signal gets flatter after the measured molar ratio of 1.5.

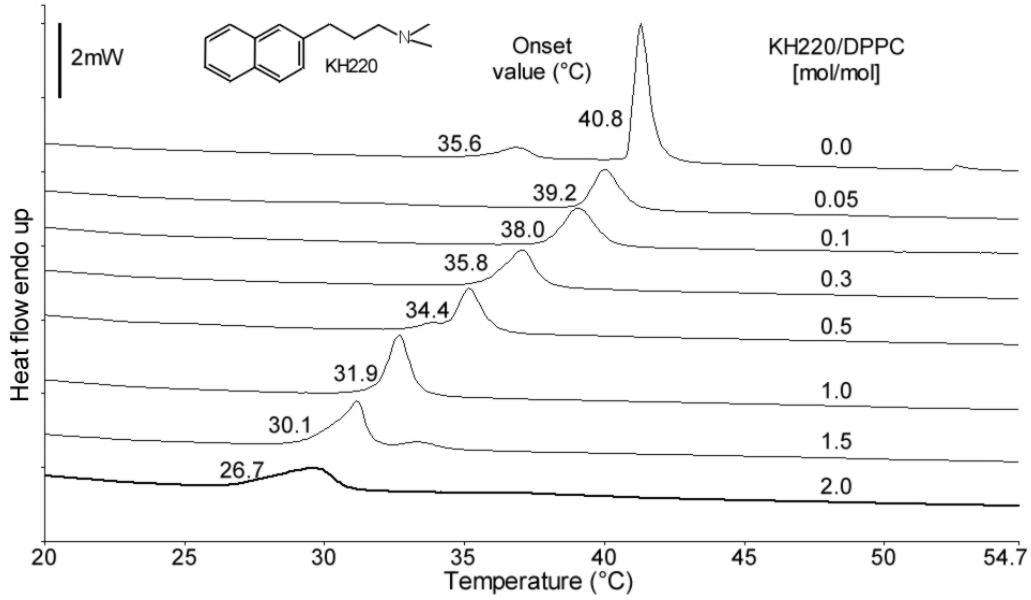


Figure 3-46: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH220 in the indicated molar ratios. A detergent effect was evident at a molar concentration of substance to phospholipid of about 2.0.
Ordinate: endothermic heat flow. As shown, each division represents 2mW. ***Abscissa***: sample temperature in °C. Shown here are thermograms resulting from one of two experiments.

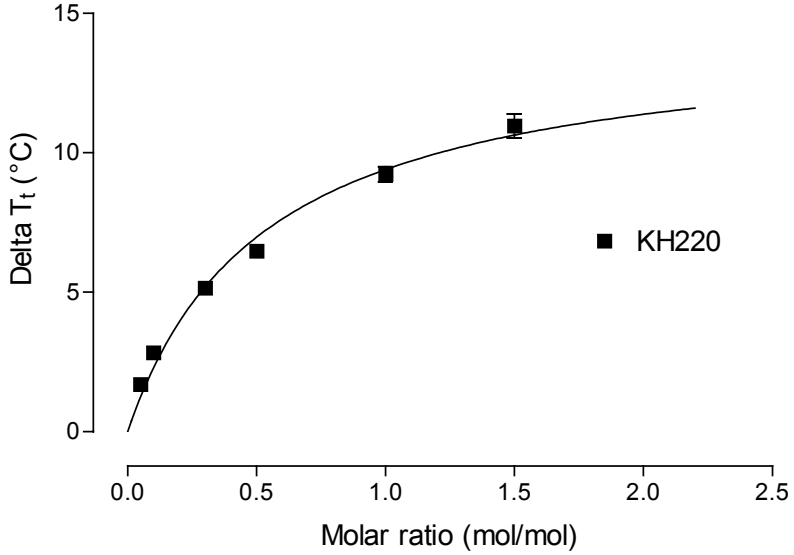


Figure 3-47: Dose-effect curve illustrating the influence of the phenylpropylamine KH220 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The value for a molar ratio of 2.0 was left out due to the shape of the peak and difficulty in determining the exact onset value of the signal. The extrapolated curve produced a ΔT_t plateau value of about 12°C.

3.5.2.9 CK84

Thermograms resulting from an experiment carried out with this substance are shown in the diagram below. No hints of a detergent effect could be observed to a substance/phospholipid molar ratio of 2.0

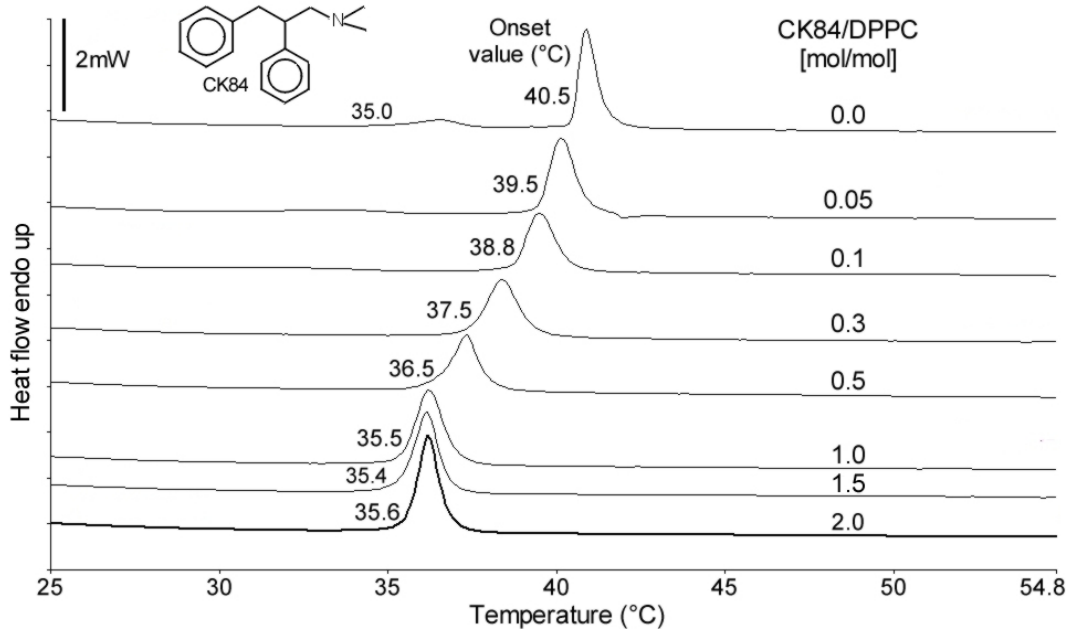


Figure 3-48: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance CK84 in the indicated molar ratios.
Ordinate: endothermic heat flow. ***Abscissa***: sample temperature in °C. Shown here are thermograms resulting from one of two experiments. The onset values for the last three molar ratios are similar.

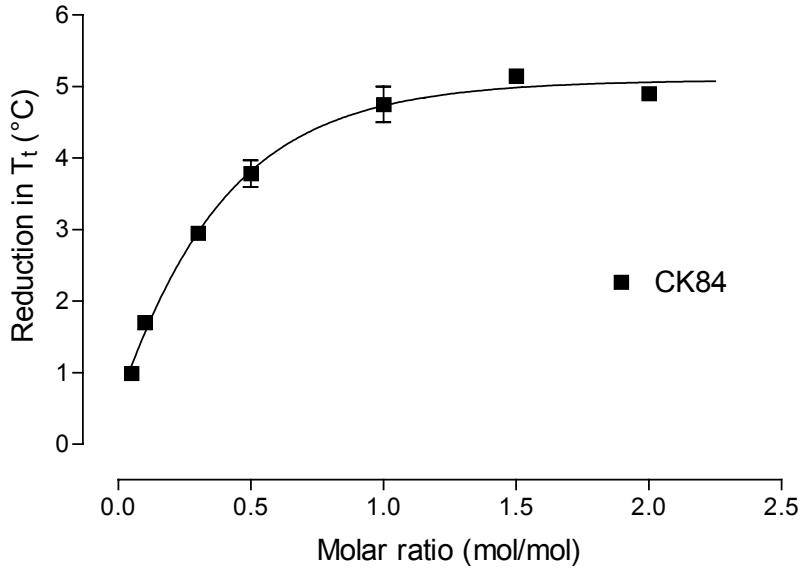


Figure 3-49: Dose-effect curve illustrating the influence of the phenylpropylamine CK84 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. Notice the divisions of the scale. The maximum reduction in T_t is relatively small. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The extrapolated curve produced a ΔT_t plateau value of slightly over 5°C.

3.5.2.10 CK41

The signals from one of the experiments carried out with this substance are shown below. A milky white suspension was observable with no sign of a detergent effect to a molar ratio of 2.5.

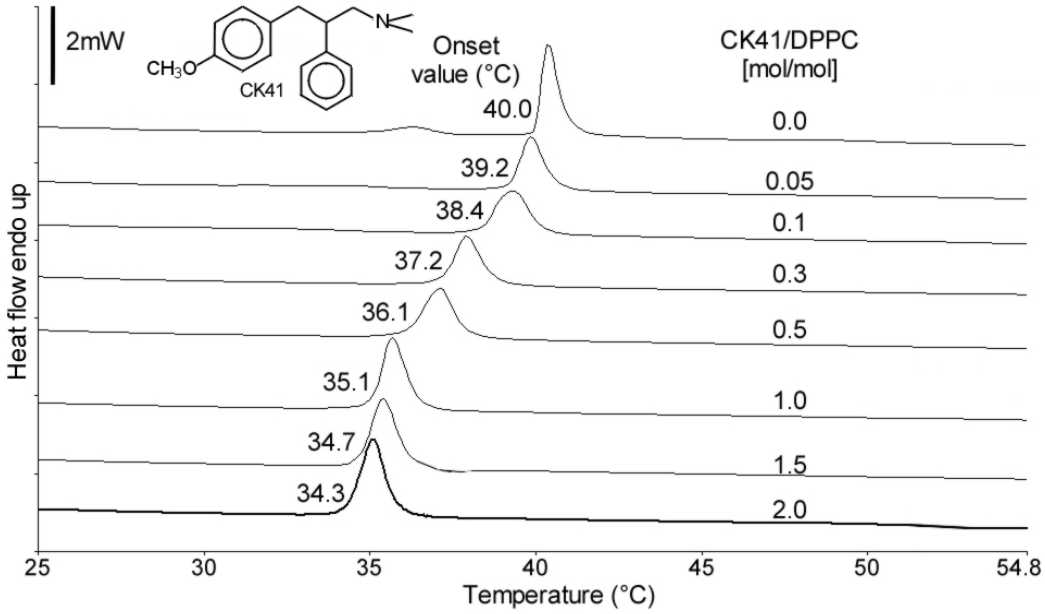


Figure 3-50: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance CK41 in the indicated molar ratios.***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

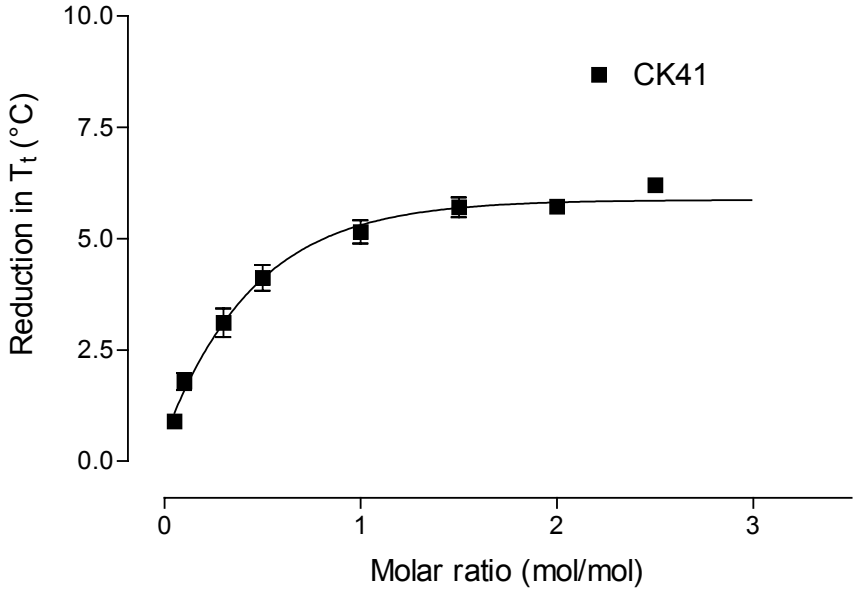


Figure 3-51: Dose-effect curve illustrating the influence of the phenylpropylamine CK41 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The last value results from just one of the two experiments. The extrapolated curve produced a ΔT_t plateau value of about 6°C.

3.5.2.11 CK94

Thermograms resulting from an experiment carried out with this substance are shown in the diagram below.

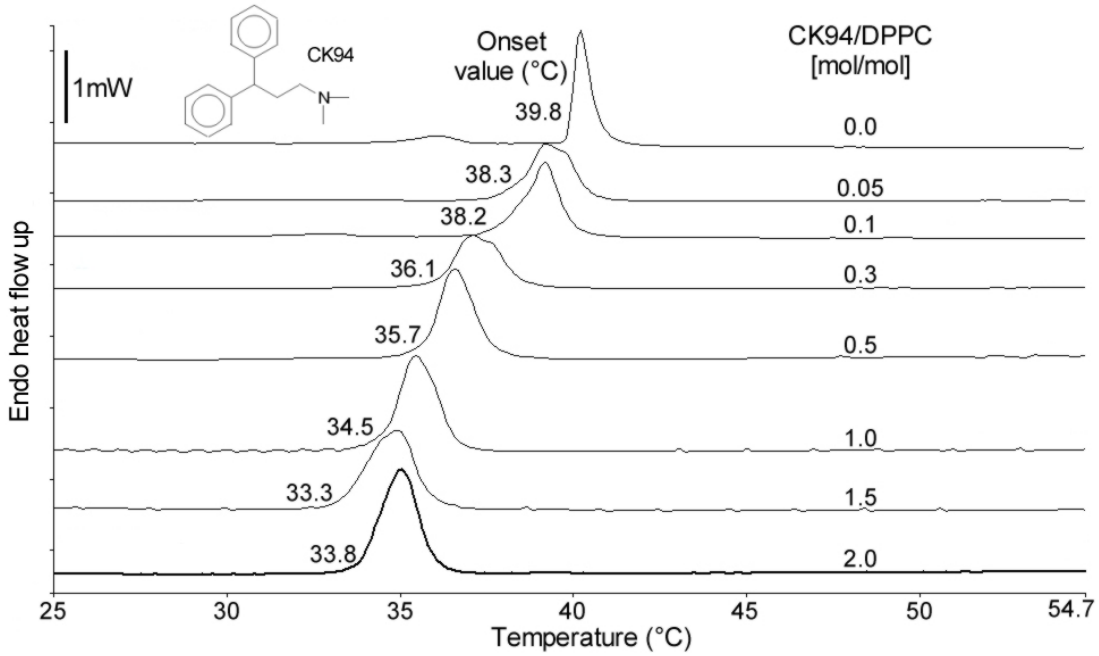


Figure 3-52: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance CK94 in the indicated molar ratios. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

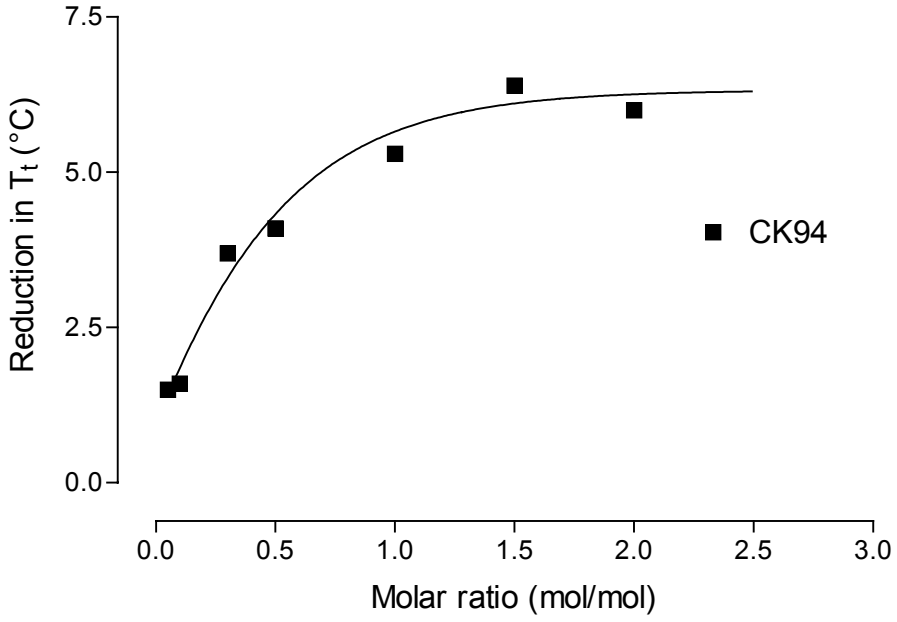


Figure 3-53: Dose-effect curve illustrating the influence of the phenylpropylamine CK94 on the phase transition temperature of DPPC liposomes.
Ordinate*:* reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The extrapolated curve produced a ΔT_t plateau value of about 6.5°C.

3.5.2.12 CK92

Results obtained from measurements using CK92. The peaks all have very broad bases with resulting in difficulties in determining the actual onset temperature. No peak is evident at a molar ratio of 1.0, with the detergent effect ensuing at a molar ratio between 0.5 and 1.0 and ΔT_t appears to be very high.

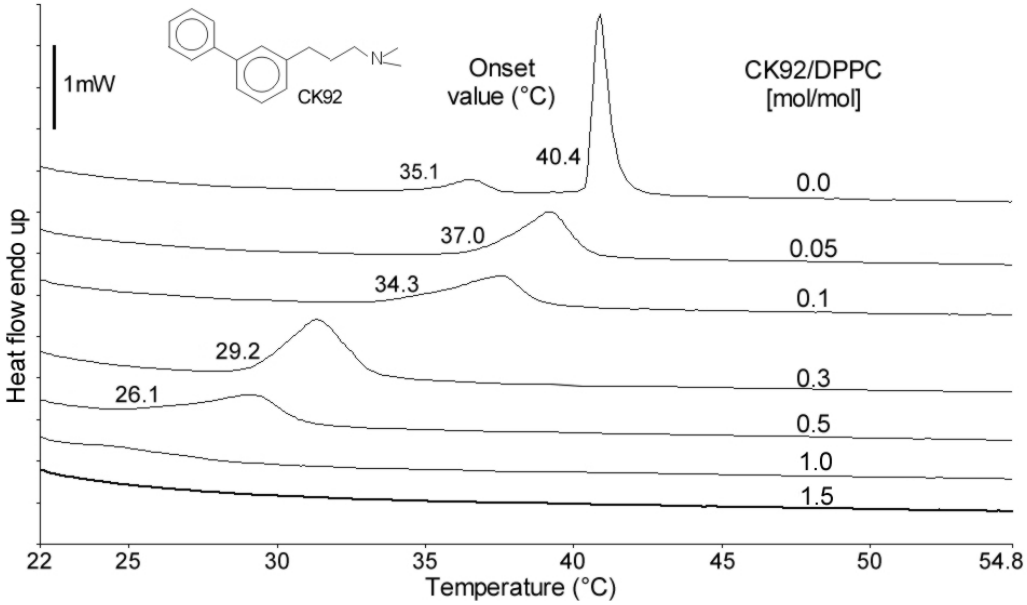


Figure 3-54: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance CK92 in the indicated molar ratios. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

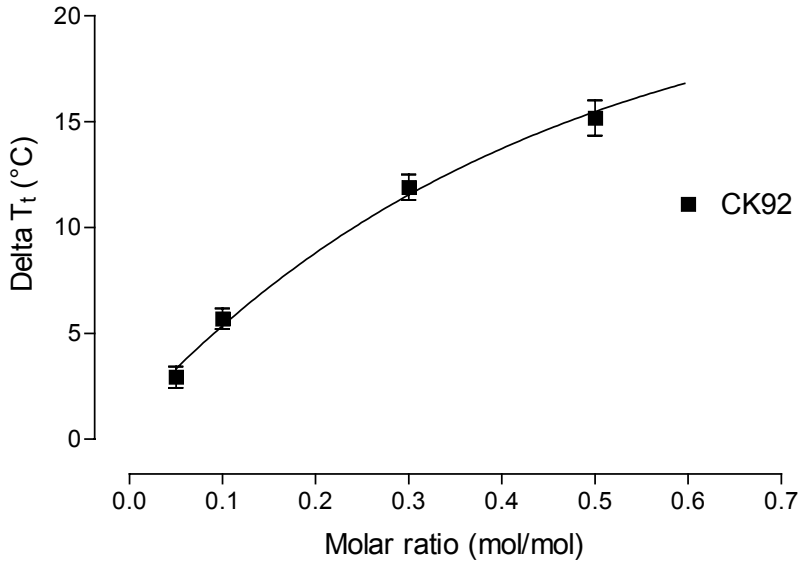


Figure 3-55: Dose-effect curve illustrating the influence of the phenylpropylamine CK92 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. Due to the shape forms and the early ensuing of the detergent effect, it was difficult to get values right to the plateau stage of the curve.

3.5.2.13 KH204

The reduction in T_t brought about by this substance is shown in the thermograms below. A dose effect curve is also shown in figure 3-57. A detergent effect was observed at a molar ratio of about 1.0.

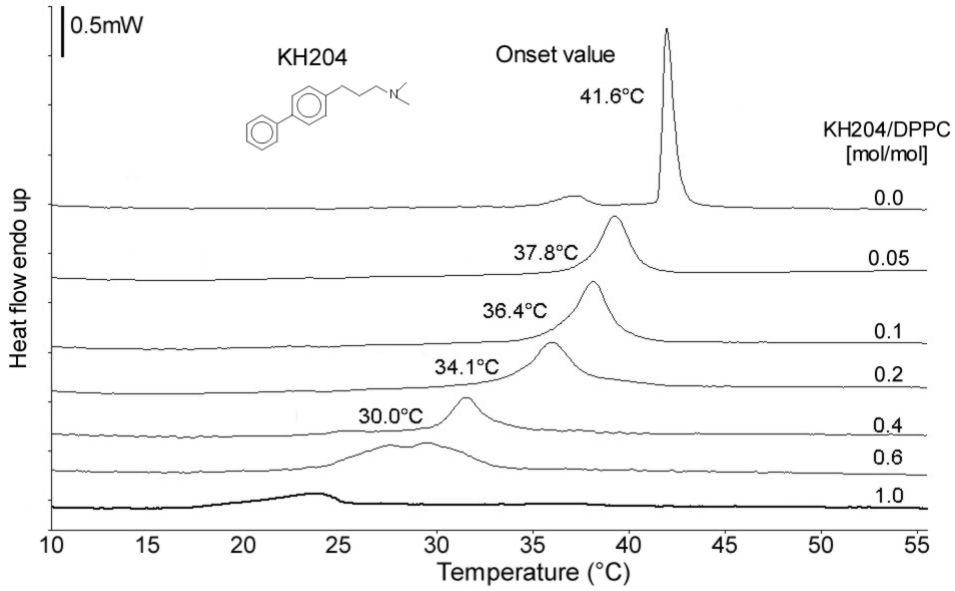


Figure 3-56: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH204 in the indicated molar ratios.
Ordinate: endothermic heat flow. ***Abscissa***: sample temperature in °C. The signals lose their symmetry at higher molar ratios, with multiple peaks making the determination of the exact onset temperature difficult. The situation is similar to that of CK92, shown in figure 3-56.

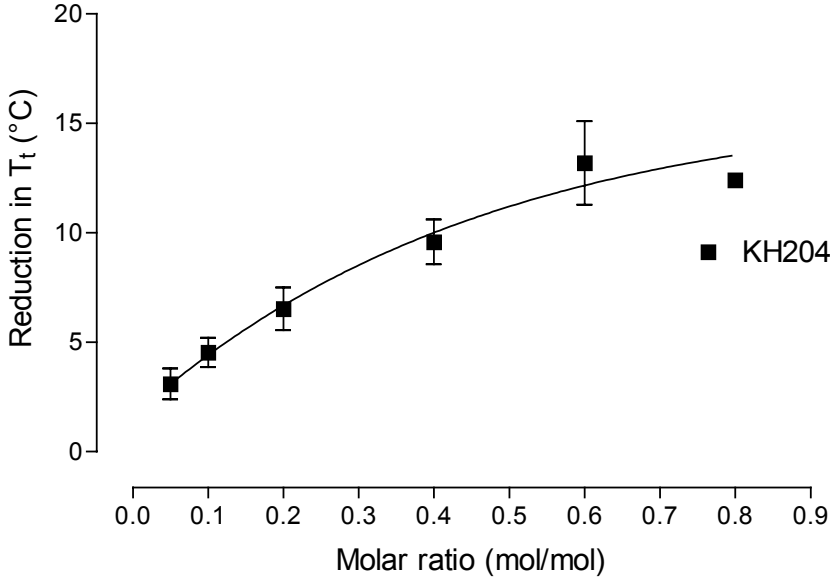


Figure 3-57: Dose-effect curve illustrating the influence of the phenylpropylamine KH204 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. The value at a molar ratio of 0.8 resulted from just one of the experiments.

3.5.2.14 KH241

Here, liposome dispersions could not be prepared at molar ratios above 1.0. Attempts to produce liposome dispersions beyond this molar ratio resulted in colourless solutions: the detergent effect.

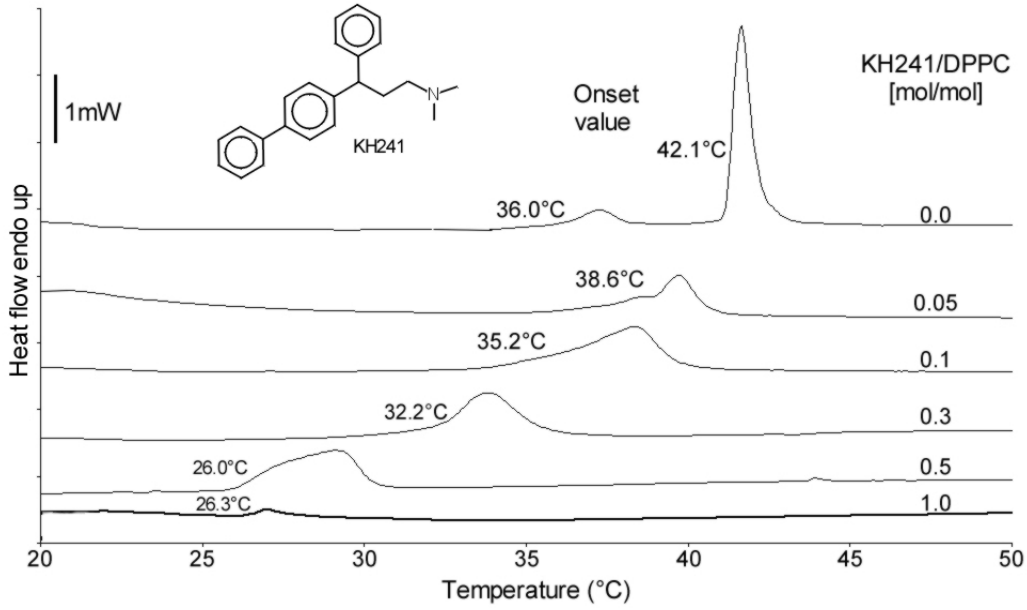


Figure 3-58: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance KH241 in the indicated molar ratios.
Ordinate: endothermic heat flow. As shown, each division represents 1mW. ***Abscissa***: sample temperature in °C.

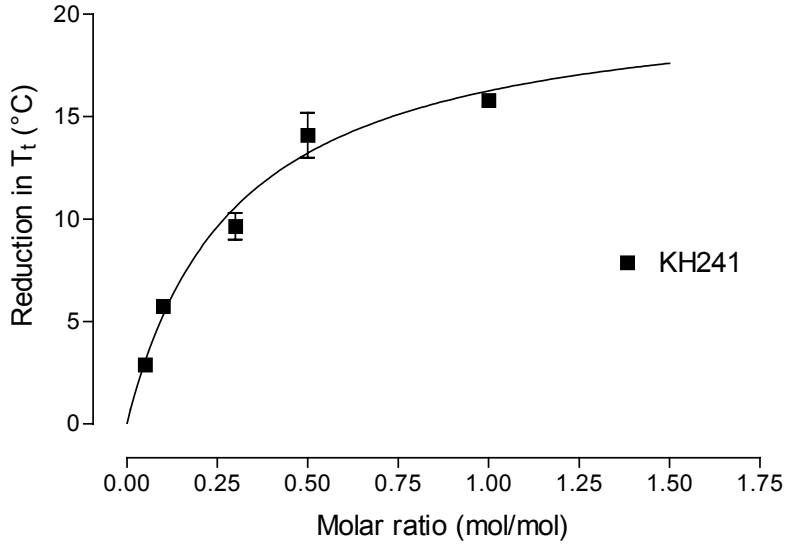
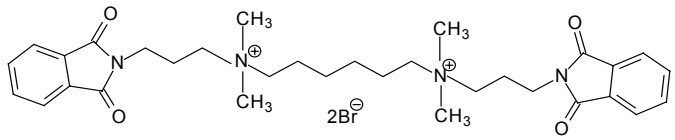


Figure 3-59: Dose-effect curve illustrating the influence of the phenylpropylamine KH241 on the phase transition temperature of DPPC liposomes.
Ordinate*:* Reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. Here too as was the case like with KH204 (Fig. 3-56), determining the onset temperature was particularly difficult at higher molar ratios due to the shapes of the signals.

3.5.3 Further compounds

3.5.3.1 Muscarinic acetylcholine receptor modulators

3.5.3.1.1 W84



Results from a representative experiment carried out with the M_2 allosteric modulator W84 are shown below in figure 3-60. The structural formula of the substance is also shown.

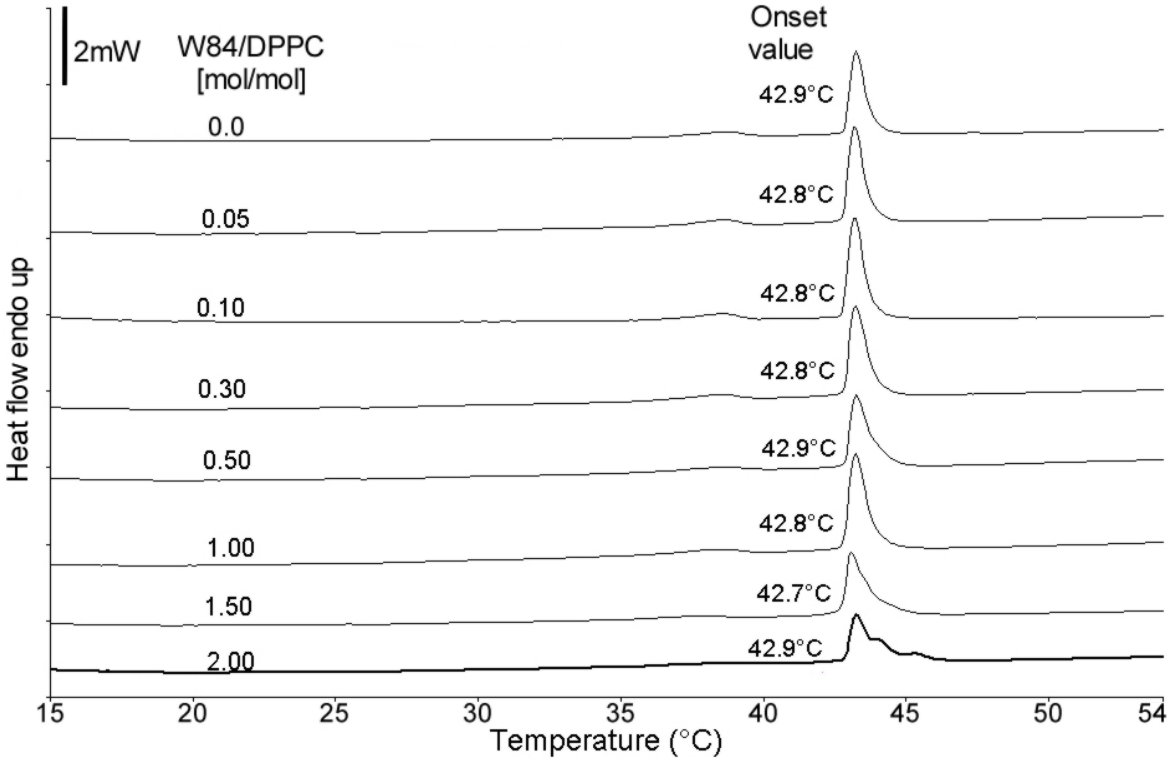


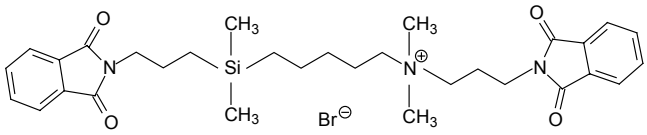
Figure 3-60: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance W84 in the indicated molar ratios. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

On the next page is a table of the T_t values measured from the use of the substance W84 in DPPC liposomes in the corresponding molar ratios in three separate experiments.

Experiments conducted with W84-DPPC liposomes				
	Sampe 1	Sample 2	Sampe 3	
Molar ratio	T_t (°C)	T_t (°C)	T_t (°C)	Average T_t ± SEM (°C)
0.00	42.7	43.1	42.9	42.9 ± 0.12
0.05	42.8	43.1	42.8	42.9 ± 0.17
0.10	42.9	43.2	42.8	43.0 ± 0.12
0.20	42.9		42.8	42.9 (± 0.5)
0.50	42.9	43.2	42.9	43.0 ± 0.10
1.00	42.8	43.1	42.8	42.9 ± 0.10
1.50	42.8	43.0	42.7	42.8 ± 0.10
2.00	42.9	43.0	42.9	42.9 ± 0.03

Table 3-20: Phase transition temperature values obtained from W84-containing DPPC liposomes. The average value of T_t from all the measurements (n = 23) amounted to 42.9 ± 0.03 °C (mean value ± SEM). Compared with the values obtained using pure DPPC liposomes, the P value was 0.17 and therefore the difference in the values considered not significant.

3.5.3.1.2 TD5



The results of calorimetric measurements using liposomes containing this compound as test substance are shown below.

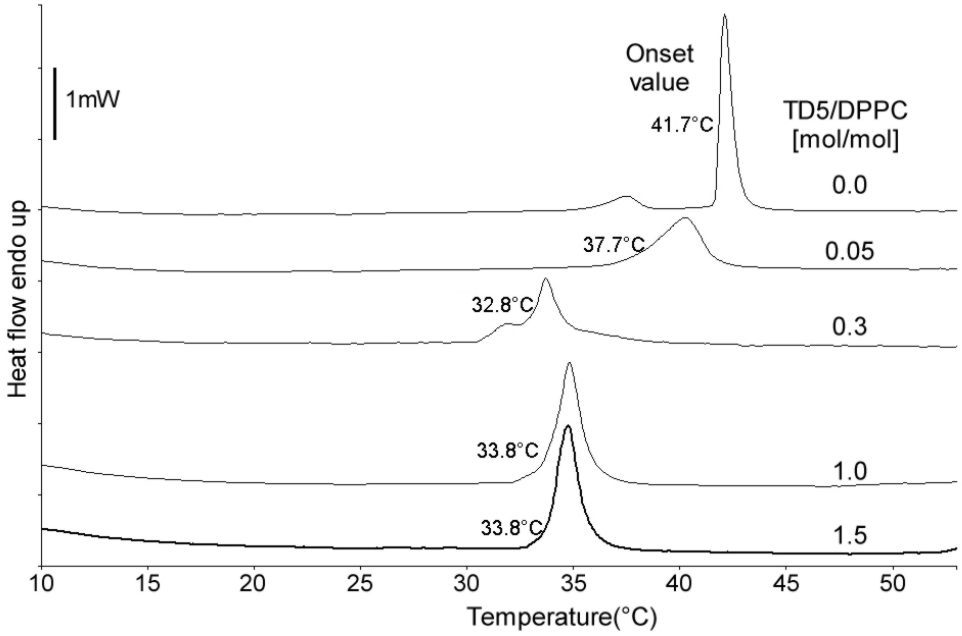


Figure 3-61: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance TD5 in the indicated molar ratios. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

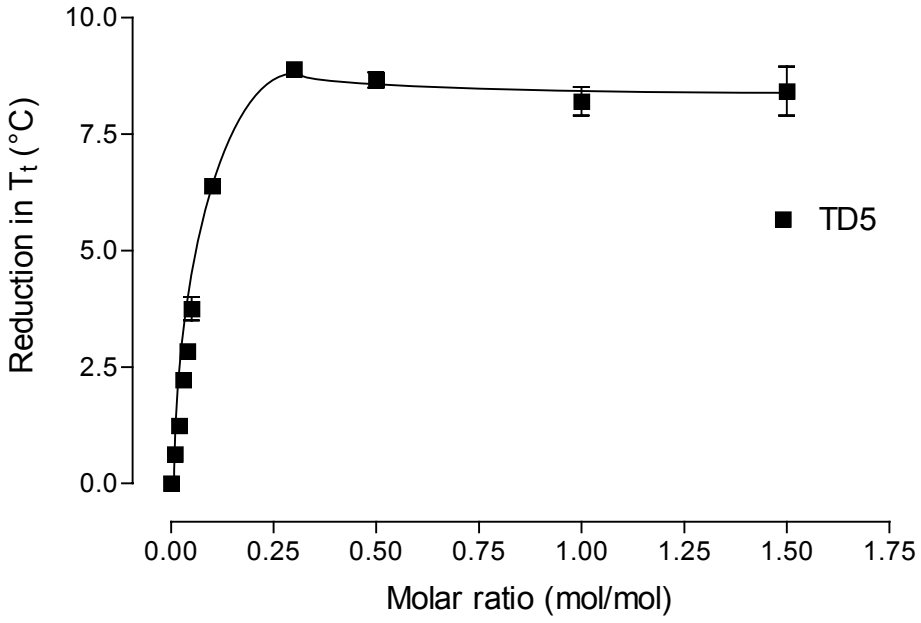
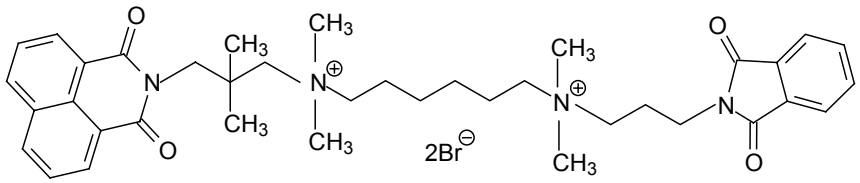


Figure 3-62: DPPC dose-effect curve of the silicon-containing TD5.
***Ordinate**:* reduction of the phase transition temperature. ***Abscissa**:* molar ratio of the test substance to DPPC. Shown here are the mean values and the standard errors of the mean from two experiments. ΔT_t attains a maximum value at about 0.25 moles of TD5 per mole of DPPC and then eventually drops marginally.

The substance differs from W84 through the replacement of a quartenary nitrogen atom with a silicon atom and a shortening of the central alkyl chain by a single methylene unit but the substance brings about a change in ΔT_t that W84 does not seem to.

3.5.3.1.3 Naphmethonium



The figure below shows the results of calorimetric measurements using liposomes containing this compound as test substance.

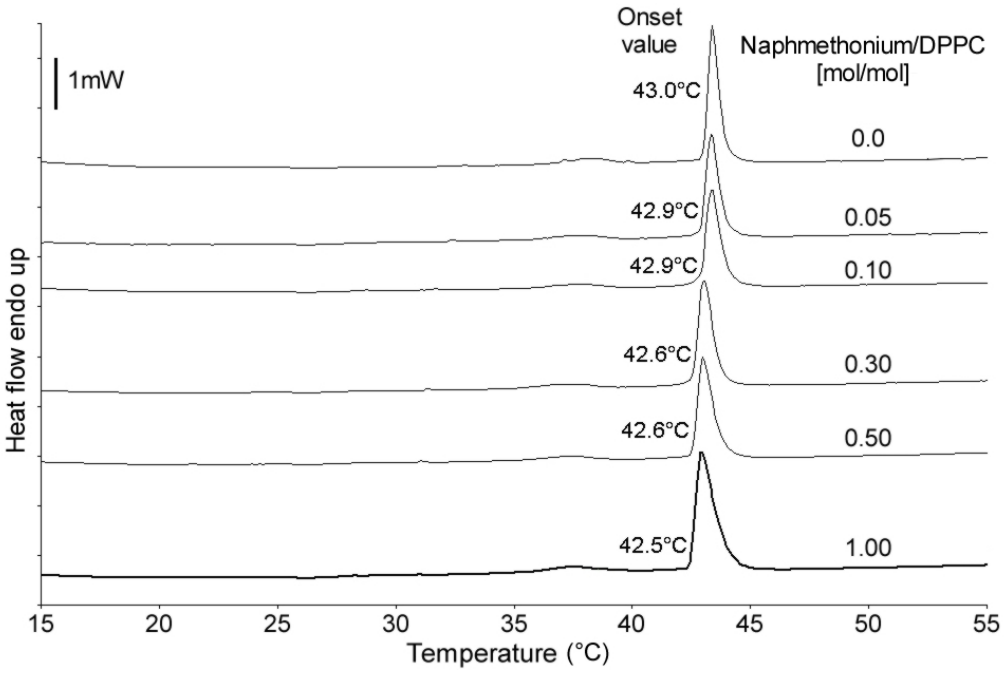


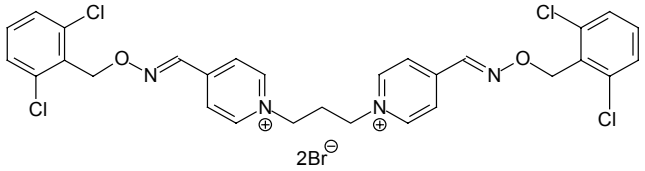
Figure 3-63: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance Naphmethonium in the indicated molar ratios. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

Experiments conducted with Naphmethonium-DPPC liposomes			
	Sample 1	Sample 2	
Molar ratio	T_t (°C)	T_t (°C)	Average T_t (°C)
0.00	43.0	43.1	43.1
0.05	43.0	42.9	43.0
0.10	42.9	42.9	42.9
0.20	42.8		42.8
0.50	42.7	42.6	42.7
0.80		42.6	42.6
1.00	42.6	42.5	42.6
1.50	43.0	42.4	42.7

Table 3-21: Phase transition temperature values obtained from Naphmethonium-containing DPPC liposomes. The average value of T_t from all the measurements (n = 14) amounted to 42.8 ± 0.22°C (mean value ± SD). The value of the SEM was 0.06. Comparing the mean T_t resulting from the other tested mole ratios with that from pure DPPC yielded a two-tailed P value <0.05, considered significant.

The difference in T_t caused by this bisammonium compound is, however, small.

3.5.3.1.4 Duo3



The results from a representative experiment are shown in the graph below.

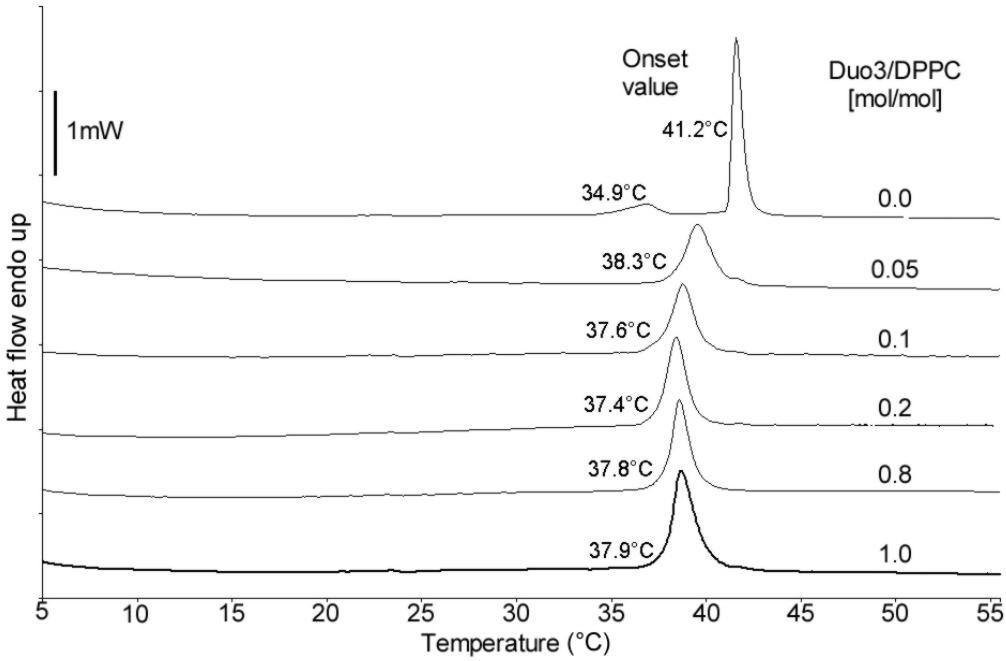


Figure 3-64: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance Duo3 in the indicated molar ratios. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

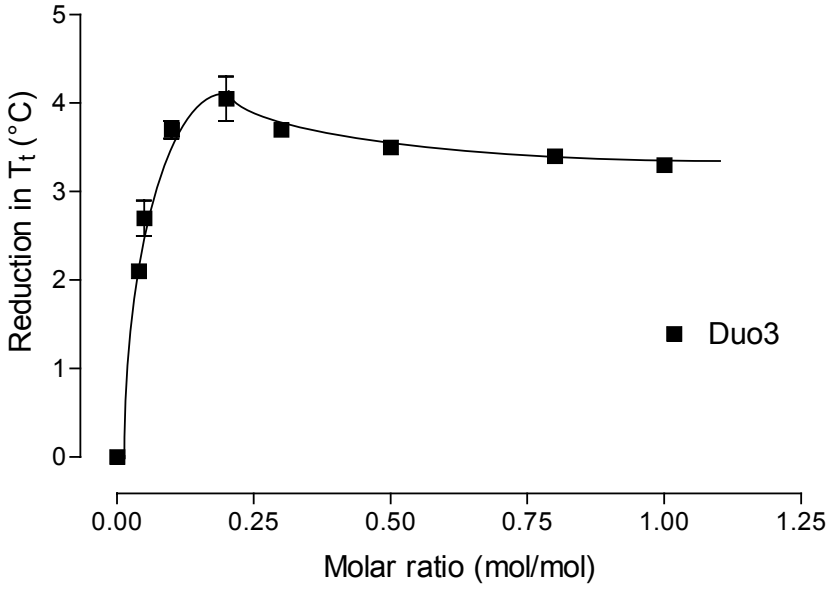
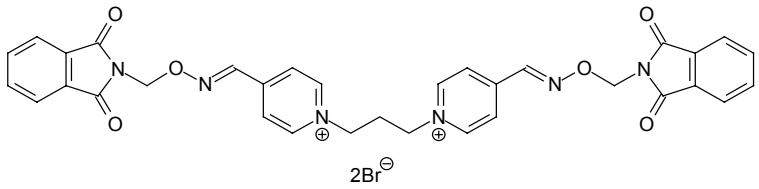


Figure 3-65: Dose-effect curve illustrating the influence of the compound Duo3 on the transition temperature of DPPC liposomes.
Ordinate*:* reduction of the phase transition temperature. ***Abscissa****:* molar ratio of the test substance to DPPC.

As with the compound TD5, an initial reduction in T_t is followed by a slight increase in the phase transition temperature with an increase in substance to DPPC molar ratio.

3.5.3.1.5 Wduo3



Results obtained from measurements with Wduo3 as test substance are shown below.

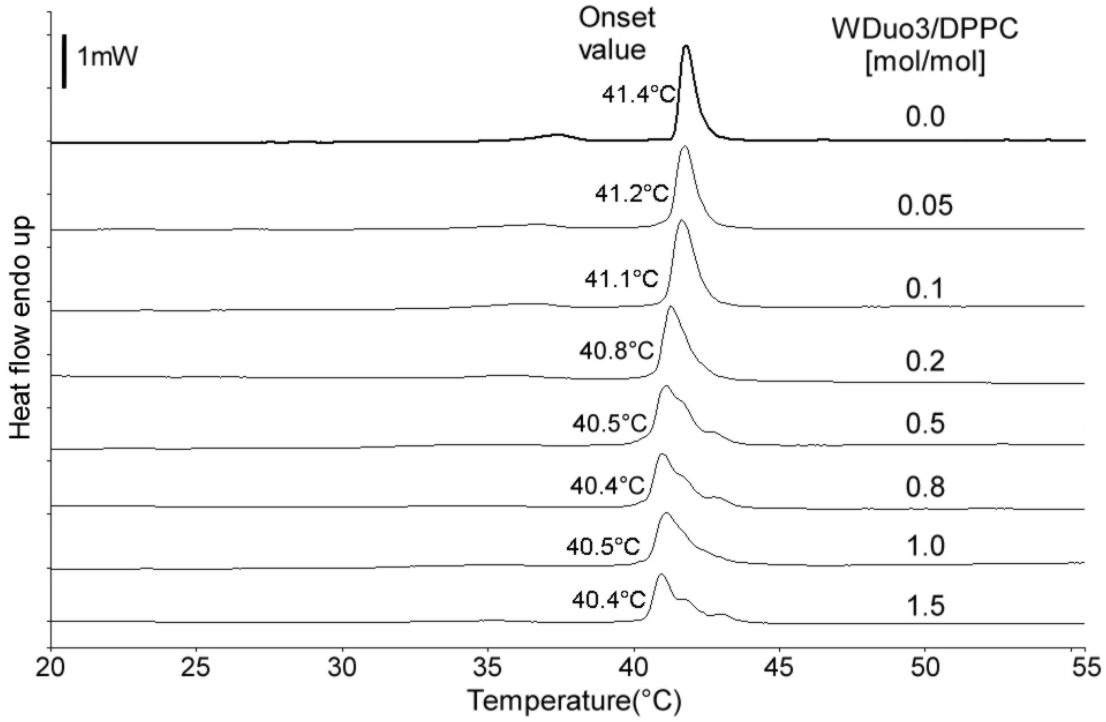


Figure 3-66: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance Wduo3 in the indicated molar ratios. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

A closer look at the thermograms reveals that the transition peaks above a molar ratio of 0.1 are not symmetrical. This was not the case with Duo3, where the peaks remained more or less symmetrical to the maximum measured molar ratio of 1.0. This is usually an indication of a superimposition of a sharp and a broad component.

Experiments conducted with Wduo3-DPPC liposomes			
	Sample 1	Sample 2	
Molar ratio	T_t (°C)	T_t (°C)	Average T_t (°C)
0.00	41.4	41.3	41.4
0.05	41.2	41.2	41.2
0.10	41.1	41.0	41.1
0.20	40.8	40.2	40.5
0.50	40.5	38.9	39.7
0.80	40.4	39.6	40.0
1.00	40.5	39.7	40.1
1.50	40.4	39.8	40.1

Table 3-22: Phase transition temperature values obtained from WDuo3-containing DPPC liposomes. The mean value of the averages in column 4 without that from pure DPPC (n = 7) amounted to 40.1 ± 0.26°C (mean value ± SEM). The resulting P value when compared to the value obtained using pure DPPC was <0.05, thus significantly different considered very significant.

A slight reduction in the T_t is noticeable from the values of the averages, a reduction which is mole-ratio dependent.

3.5.4 Substance-phospholipid interactions and enthalpy

Quantitative estimates of the heat involved in these phase transitions and the related entropy changes have previously been discussed (Phillips, M. C., Williams, R. M., Chapman, 1969). The change in enthalpy, ΔH values was initially expected to provide valuable information in this work about the differences in mode of interaction with the phospholipid membranes. However, although it was easy to determine the onset value of most of the peaks reasonably accurately, the point at which the signal deviated from the baseline was not always easy to determine, particularly as the shapes of the signals were very heterogeneous. These latter ranged from narrow to broad base and to multi-peak signals. The other factor was the different molar ratios at which the detergent effect ensued. This posed a problem, since it was not possible to consistently measure ΔH values that could be used for the comparison of the phenylpropylamines and the allosteric modulators. Therefore, this parameter was not systematically observed in the present work.

4. Discussion

Extensive research has been carried out on substances that modulate ligand binding to muscarinic acetylcholine receptors using binding studies. Mediation of signals from these seven transmembrane spanning cell surface receptors to a variety of effects is done with the help of heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins). These receptors thus belong to the super-family of G-protein coupled receptors.

The modulators are structurally very heterogeneous. While the modulator W84 binds unto the “common allosteric site” of M_2-acetylcholine receptors, there are indications from studies in recent years that point to binding of some allosters unto a separate binding site (Traenkle et al., 2003). The common allosteric site was so termed based on initial observations, in the course of experiments carried out with [^{3}H]-NMS occupied receptors, that modulators could displace one another from their respective binding in a competitive manner (Ellis and Seidenberg, 1992). Thus, the binding site was “common” to both modulators.

The introduction of an interphase model in the investigation of allosteric modulators could be an interesting complement to the already established methods. As mentioned in the introductory section, the approach is based on the abrupt change in physical properties of a phospholipid bilayer over a definite temperature range. The influence of cationic amphiphilic model substances including drug substances on artificial phospholipid bilayers has been applied by many groups to gain more knowledge about the nature of hydrophilic-hydrophobic interactions (Cater et al., 1974; Hanpft, 1987; Mohr, 1987). Using the method of differential scanning calorimetry, with phase transition temperature and the corresponding associated change in enthalpy as measuring parameters and the help of the resulting thermograms, research has been carried out on the extent to which test substances react with phospholipids (Cater et al., 1974; Surewicz and Leyko, 1981; Kursch et al., 1983; Hanpft and Mohr, 1985; Girke et al., 1989; Borchardt et al., 1991; Mohr and Struve, 1991).

The two phospholipids used in this work are DPPA and DPPC (section 2.1.2). As shown in the results (sections 3.1.1 and 3.1.2), thermograms resulting from the measurement of pure DPPC liposomes contain a pre-transition and a major transition peak (Fig 3-1), while those resulting from the measurement of pure DPPA liposomes possess just a single, main-transition signal.

4.1 The pre-transition peak

Ladbrooke and Chapman associated the pre-transition peak with an increase in mobility of the polar head portions of the phospholipids (Ladbrooke and Chapman, 1969). This has been supported by nuclear magnetic resonance (NMR) spectroscopic studies, which showed that a modification of motion of the polar group occurs prior to the main endothermic transition (Chapman et al., 1969; Ladbrooke and Chapman, 1969; Veksli et al., 1969; Chapman and Chen, 1972). Veksli et al. (1969), came to the conclusion that in aqueous solution, on warming, the first 4 to 5 water molecules form an immediate hydration layer around the head groups and so contribute in loosening the lattice formed by the latter before the transition. A rearrangement of the water molecules associated with the polar head groups occurs at the main phase transition as is shown by deuterium magnetic resonance using magnetic resonance spectroscopy (Salsbury et al., 1972).

Further investigation of the pre-transition of DPPC and other phospholipids has confirmed that the pre-transition represents an expression of structural changes in the head group (Janiak et al., 1976). A similar interpretation of the pre-transition has been put forward by Lee (Lee, 1975a). Based on conducted experiments, he found that the formation of vesicle aggregates in phospholipid dispersions could be prevented through multivalent cations. In the case of DPPC, he postulated that above the pre-transition, the zwitterionic choline-phosphate moiety possesses a conformation in which the cationic $N(CH_3)_3$-residue stretches outwards and thus determines the surface charge, a situation that can also be brought about by the interaction with these multivalent cations. Below the pre-transition on the other hand, the total head group lies parallel to the surface. This results in a mosaic-like array of dipoles so that the overall effect is the absence of a net outward charge, easing the aggregation of the vesicles. The absence of a pre-transition peak in thermograms from DPPA liposomes testifies to the factors responsible for its origin in DPPC thermograms. The pre-transition signal reacts in a highly sensitive manner to manipulations in the head group region (Chapman and Urbina, 1974). Based on the findings of the various groups, it is not surprising that substances that interact with the polar head groups of phospholipids have an effect on the pre-transition peak.

In the course of this work, substances were tested that interacted in varying degrees with the phospholipid membranes. The pre-transition signal normally disappeared from the thermogram as a result of these interactions. In cases where there was a

measurable interaction of test substance with the phospholipid membranes, the pre-transition signal usually disappeared even at the lowest measured ratios such as 0.02 for propranolol (Fig. 3-29), concentrations at which only a minor change could be observed in the main transition signal.

Despite the highly sensitive manner in which the pre-transition peak reacts to head group changes, disappearing at very low substance-phospholipid molar ratios, it has been found that a substance induced shift in the pre-transition temperature of DPPC can occur and can be observed. From systematic investigation of n-alcohols on the pre-transition of DPPC, Veiro et al. (1987) found that while these remove the pre-transition above a critical concentration, short-chain n-alcohols decrease the pre-transition temperature (T_p) at concentrations below the critical concentration. The longer the aliphatic chain length of the n-alcohol (up to butanol) the greater the decrease in the pre-transition temperature, and the lower the concentration necessary to remove the pre-transition (Veiro et al., 1987).

O'Leary et al. (1986) observed a dramatic increase in pre-transition temperature of DPPC with trans-tetradecenol. They interpreted the results in terms of a reduction in gel phase chain tilt and changes in the ease of acyl chain trans-gauche isomerization introduced by the alcohol, and the consequent effects of the changes on the pre-transition and the gel to liquid crystalline phase transition (O'Leary et al., 1986). Thus, a shift in T_p is possible under certain circumstances.

4.2 The main transition peak

The thermograms resulting from measurements with simple lipid systems usually contain clear peaks. A number of properties of the phospholipid used tend to influence the shape and size of the signals and the temperature at which these appear. Of particular importance are the volume and charge of the phospholipid head group, the chain lengths and degree of saturation of the fatty acyl chains in the molecule.

The zwitterionic character of the DPPC head group existing under the experimental conditions has a dual effect: firstly, it promotes the formation of hydrogen bonding and it would be expected that these lead to relatively high T_t values. On the other hand, the large head groups lying parallel to the surface tend to reduce the likelihood of close packing of the hydrocarbon chains, and so limit interaction through van der Waals forces of attraction due to the chains' distances apart (Chapman and Urbina, 1974) especially in the regions close to the headgroups. This is apparent in the work of

Hanpft where the values of the onset temperatures of liposomes carried out with dipalmitoylphosphatidylcholine, DPPC and dipalmitoylphosphatidylglycerine, DPPG at a pH value of 6 appear at the respective temperatures of 41°C and 42°C (Hanpft, 1987). DPPC is zwitterionic and is neutral at this pH. DPPG is negatively charged, similar to DPPA at the same pH value. The sole difference between the two latter substances under these circumstances lies in the size of their head groups. The DPPA molecules tend to be more densely packed together due to the smaller size of their head groups, hence the much higher T_t value of DPPA of about 63°C. The main transition peak tends to be associated with the polar head group and the acyl chain mobility. The pH-dependence of the phase transition temperature of various phospholipids is supported by the work of many groups (Boggs, 1986; Achiriloaie et al., 1999). The interaction of the test substances with the DPPA molecules apparently annuls the specificity brought about by the negative charge of the head groups (Hanpft and Mohr, 1985). But the heterogeneous nature and in some cases mole ratio specific multi-peak signals from the various substances can hardly be explained by charge neutralisation alone. The above-mentioned sizes of the head groups as well as dipoles existing temporarily may also play a role in the observed signals.

As explained above, beyond the pre-transition, the lipid chains change from a tilted condition to an orientation with chains perpendicular to the plane of the lamellae (Tardieu et al., 1973). The presence of certain foreign substances in the membrane may also alter the orientation so that it becomes easy for molecules lying perpendicular to the surface to interrupt the interaction of the acyl chains with each other. This leads to a breakdown of the crystalline gel arrangement at a lower temperature.

During the phase transition, the hydrocarbon chains coexist in gel and liquid crystalline forms (Chapman and Urbina, 1974). According to Cater et al. (1974), the resultant phase transition temperature measured in the presence of test substance is essentially not an average of two extreme temperatures as is often the case when two similar lipids are mixed.

Although a reduction in T_t is often the case, T_t can indeed be increased, as was observed with DPPA, by the presence of certain test substances in specific molar ratios. This was the case with the test substances Duo3 and WDuo3. Such an increase was also observed by Mohr with the amino-glycoside antibiotic gentamicin (Mohr, 1987). In similar cases, a complex has been observed within certain substance-

phospholipid combinations (Cater et al., 1974). Although not observed with the substances tested in this work, cases have been reported where an increase in the T_t of the DPPC main transition is observed (Eliasz et al., 1976). Investigating the interactions of straight chain alcohols and acids with DPPC, they observed that n-alcohols and n-monocarboxylic acids containing 12 or more carbon atoms raise the main lipid phase transition whilst those molecules containing 10 or less carbon atoms lower this transition temperature

The discontinuity in the signals observed in DPPA experiments could be explained by the existence of a two-component system: domains with complexes formed between the test substance and the DPPA molecules in a fixed ratio and domains with pure DPPA molecules. At low molar ratios of substance to DPPA, the pure DPPA domains predominate. At higher molar ratios, the initially low concentration of substance-DPPA complex, whose composition does not change, becomes the sole component of the system. Hence the substance-induced peak gets bigger without a change in onset temperature (Hanpft and Mohr, 1985).

Depending on the specific nature of the complex formed with DPPA, the ratio of substance-to-phospholipid and the depth of penetration of the test substance, different onset temperatures and peak forms are observed for the various substances. A possible explanation for the existence of the multipeak signals is a conformational change in the species involved, forming different complexes at different temperatures. These multi-peak signals were only observed with the structurally more complex substances and did not occur with the simpler phenylpropylamine derivatives.

With DPPC, clusters of gel and liquid crystalline lipids coexist. Due to the neutral nature of the zwitterionic DPPC head group at the experimental pH, it appears similar complex formation as is the case with DPPA is unlikely. Although Eliasz et al. pointed out that the phase diagram of stearyl alcohol in the DPPC-water system shows the formation of lipid-alcohol complexes (Eliasz et al., 1976), somehow, the formation of distinct peaks that could be ascribed to the existence of domains containing pure DPPC and those with DPPC and test substances in definite ratios as can be observed with DPPA was lacking in this work. There is a gradual decrease in onset temperature of the main transition due to the increased disturbance in van der Waals interactions caused by the test substances. Surewicz et al. studied the effect of phospholipid structure on the interaction between small peptides and phospholipid membranes by high-sensitivity differential scanning calorimetry (Surewicz and Epand, 1986). The

peptides used were basic analogues of the hormone pentagastrin. These peptides split the gel-to-liquid crystalline phase transition of synthetic phosphatidylcholines into two components. For DPPC, one component remained at the temperature corresponding to that of pure lipid and the other one was shifted towards higher temperatures. With increasing peptide concentration there was a gradual increase in the enthalpy of the high-temperature component at the expense of the low-temperature one, and there was also an increase in the total enthalpy of the transition. Thus peak splitting is not a phenomenon limited to substance interactions with DPPA.

4.3 Systems containing more than one type of phospholipid

It has been shown that a mixture of lipids from the class lecithin with dissimilar chain lengths leads to a continuous series of solid solutions below the T_t line, and to the occurrence of co-crystallization. No great change occurs in the range of the thermal transition as a result of the mixing. On the contrary, mixing lipids of the lecithin and phosphatidylethanolamine classes with the same chain length leads to a considerable increase in transition range. Clusters of gel and liquid crystalline lipids probably coexist within this temperature range (Chapman and Urbina, 1974)

Experiments were performed by Hanpft with liposome dispersions containing a mixture of DPPC and DPPA in equal molar ratios (Hanpft, 1987). The resultant thermogram showed a signal containing two broad peaks with the signal lying between the peaks that would normally be produced by the individual phospholipids alone. Chapman et al. used lecithin and a phosphatidylethanolamine. The result was a single peak with a relatively broad base at equimolarity. It seems there are fundamental differences in the manner in which the phospholipids from different classes interact with each other. The interaction of test substances with phospholipids would therefore be best-characterised using experiments containing a single phospholipid, as was the case in this work.

4.4 Comparison of the measured transition signals with those obtained by other groups

The signals resulting from measurements using pure DPPC and DPPA liposome dispersions in aqueous solution did not only differ in their shapes but also in the temperatures at which the peak of the transition signals occurred (section 3.1.1). These differed by some 20°C. This is consistent with the findings of previous groups (Chapman and Urbina, 1971; Cater et al., 1974). The onset temperatures were

comparable with those obtained by these groups, with the DPPC signal lying between 41 and 42°C (Kursch et al., 1983; Hanpft, 1987), and the main DPPA signal appearing at an average temperature of about 63°C. The average values obtained in this work were 41.5°C for DPPC and 63°C for DPPA respectively.

4.5 Effects of test substances on DPPA liposomes

4.5.1 Effects of propranolol

Studies show that the propranolol partition coefficient in negatively charged membranes of vesicles is about 20 times higher than in neutral PC membranes. The preferential interaction with acidic phospholipid membranes was also confirmed through multiple methods including the spin-labelling method (Surewicz and Leyko, 1981). This explains the observed substantial reduction in T_t of DPPA to a value of about 26°C in this work, a value that conforms with that obtained in experiments carried out earlier (Hanpft, 1987). This indicates an intercalation of the substance into the phospholipid acyl groups at the very least. Studies have been carried out with propranolol to investigate its effects on biomembranes. Jutila et al measured the detachment of a cationic peripheral membrane protein, cytochrome c (cyt c) from liposomes by propranolol as well as gentamicin and lidocaine. These substances showed different efficiencies in dissociating cyt c. These results are likely to reflect differences in the contributions of the electrostatic interactions and hydrophobicity of the test substances to the drug-lipid interaction (Jutila et al., 1998). This indicates that propranolol, as well as other drug substances, can manifest other membrane effects at certain molar concentrations.

4.5.2 Effects of phenylpropylamines

Systematically varying the substituents brought on the parent compound KH210 was necessary in establishing a relationship between the spatial arrangement of the molecules and their ability to interact with the bilayer and also the extent to which they did so. Although these substances have a skeletal structure in common, the effects they manifest on the phase transition temperature of DPPA liposome dispersions varied considerably. A look at the bar chart on the next figure (Fig. 4-1) gives an idea of the variation observed. The patterns have been chosen to simplify the recognition of the various categories of substances.

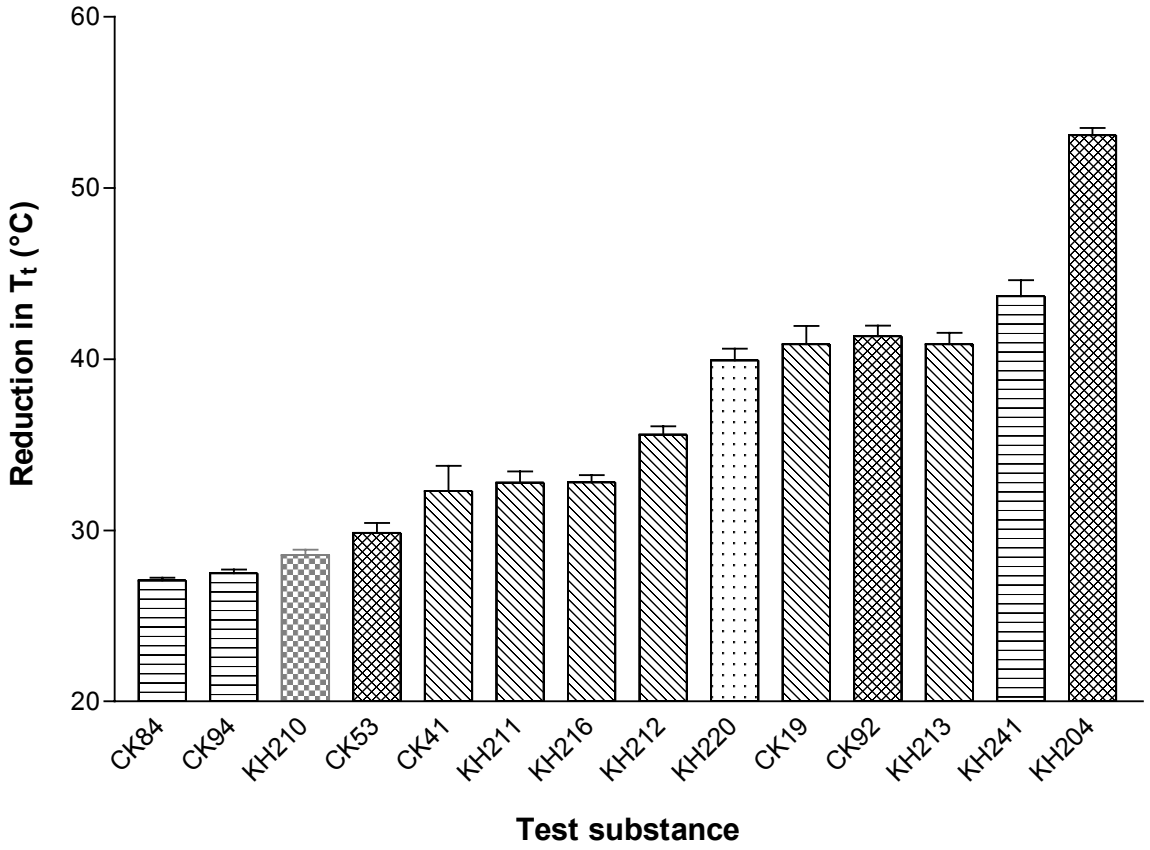


Figure 4-1: Bar chart showing the ΔT_t obtained from calorimetric measurements performed using DPPA liposomes containing the indicated test substances. ***Ordinate***: reduction in phase transition temperature. ***Abscissa***: test substance. The error bars represent the standard deviation of a minimum of 5 values (Table 3-17). The ΔT_t were generally independent of the applied substance to phospholipid molar ratio. The fill patterns were chosen to depict the substance groups as follows: bars filled with horizontal lines represent diphenyl substances. Those were CK84, CK94, KH241. Bars with a criss-cross fill pattern represent biphenyl compounds: CK53, CK92 and KH204. Compounds with alkyl substituents in para position are represented by bars filled with slanting lines. Compared to the parent compound KH210, the ΔT_t induced by the substances CK53, CK84 and CK94 were not significantly different ($p>0.05$) from that of KH210, while that brought about by all the other substances was extremely significant ($p<0.001$). In each case, a normality test was performed for the values obtained for each test substance to determine if they formed a normal distribution. All substances passed the normality test.

4.5.2.1 KH210 and compounds with alkyl substituents in para-position

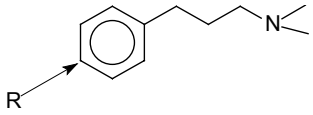
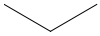
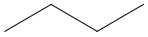
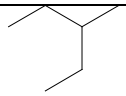
Parent compound KH210 (lop P=1.50; ΔT_t=28.6 ± 0.10°C (mean value ± SEM)) showing position in which substituents are bonded 						
	KH211	KH212	KH214	KH213	CK19	KH216
R						CH_3O
log P	2.00	2.45	2.75	2.89	3.68	1.48
ΔT_t ± SEM	32.8 ± 0.26	35.7 ± 0.20	36.8 ± 0.33	40.9 ± 0.25	40.9 ± 0.04	32.8 ± 0.13

Figure 4-2: Summary table showing the structures of the test substances containing alkyl substituents in the para-position of the phenyl ring. A measure of the hydrophobicity in terms of the logarithm of the octanol-buffer partition coefficient (log P) of the substances is included. ΔT_t values are also included to facilitate direct comparison. The parent compound had a log P value of 1.50. These values were measured using reverse-phase High-Performance Liquid Chromatography (HPLC). (Log P values from C. Klein, I999). SEM: standard error of the mean. Please refer to table 3-17 for the values considered in determining ΔT_t.

As mentioned in the legend in figure 4-1, the bars representing these compounds have a pattern with slanting lines.

The parent compound KH210, despite its simple molecular structure brings about a phase transition temperature reduction of 29°C. The derivative KH211 having a methyl group in the para position causes a further reduction of some 4°C, a value which is significantly different from that brought about by KH210.

The next derivatives KH212 and KH214 reduce T_t by similar amounts. They possess residues that are similar in length. These substances with an ethyl and isopropyl substituent respectively reduce T_t further by some 7°C. Although there is no significant difference between the ΔT_t that they both bring about, the values differ significantly from that of KH210.

KH213 and CK19 have similar activities. Like is the case with KH212 and KH214 above, their ΔT_t values do not differ significantly from each other but do so when compared with ΔT_t of the parent compound KH210. The isopentyl residue has a similar effect to the isopropyl rest.

4.5.3 Compounds with further phenyl groups

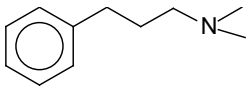
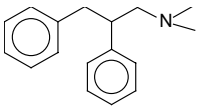
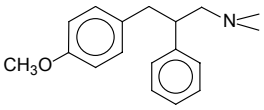
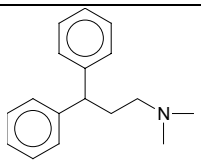
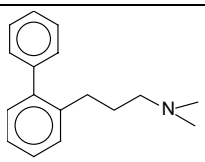
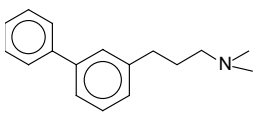
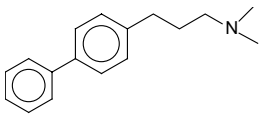
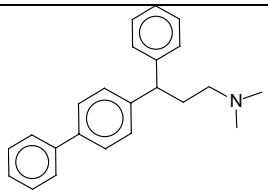
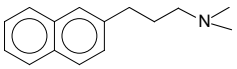
Parent compound KH210 				
	CK84	CK41	CK94	CK53
				
log P	2.27	n.d.	2.35	2.72
ΔT_t ± SEM	27.1 ± 0.07	32.3 ± 0.44	27.5 ± 0.09	29.8 ± 0.22
	CK92	KH204	KH241	KH220
				
log P	3.03	3.16	3.36*	2.62*
ΔT_t ± SEM	41.4 ± 0.26	53.1 ± 0.16	43.7 ± 0.33	39.9 ± 0.22

Figure 4-3: Summary table showing the structures of the test substances containing more than one phenyl ring. Here again, the log P values (log_{10} of the octanol/buffer coefficients of the substances) were determined using HPLC by C. Klein (1999). The values marked with an asterisk taken from M. Klingmüller (1990). n.d.: not determined. SEM: standard error of the mean. Please refer to table 3-17 for the values considered in determining ΔT_t.

Some of the bars of these compounds on the chart have a horizontal striped pattern, while others have a criss-cross fill pattern as is described in more detail in the ledend of figure 4-1. The derivatives CK84 and CK94 bring about a similar reduction in T_t and the values are not significantly different from that of KH210 (Fig. 4-1). They also have similar log P values. The presence of a phenyl group close to the nitrogen atom does not seem to interfere with the interaction between the tertiary amino function of the test substance and the phosphate group of the phospholipid than its being placed one methylene group further. The activity of the parent compound KH210 lies between those of the 3,2'-biphenylamine, CK53 and the 3,3-diphenylpropylamine, CK94. Comparing the ΔT_t of each substance individually with that induced by the parent compound, there was no significant difference. Comparing the values of the two

substances, however, showed a significant difference in their reduction in phase transition temperature ($p<0.001$). The positioning of the second phenyl group in the para position apparently influences the interaction pattern of the compound with the phospholipids differently.

The substance KH204 is by far the most active of the tested model substances with the highest ΔT_t value and this value is extremely significantly different from that of the parent compound ($p<0.001$). The phenyl groups of the substance lie twisted to each other and probably penetrate deepest into the acyl groups, resulting in an unparalleled disruption of the chain packing of the hydrocarbon tails. Interaction of these latter with each other is restricted and the inner regions of the bilayer become slightly more fluid. The melting thus occurs at a lower temperature.

An additional phenyl residue in the gamma position of the propyl chain to KH204 results in the substance KH241. The substance-induced DPPA melting temperature of this compound tends to be higher than that of KH204; ΔT_t is much smaller. Thus, the voluminous KH241 does not affect the phospholipid chains in the same manner as the compound KH204. The presence of the third phenyl ring in the structure means the parts lying away from the propyl axis do not entirely lie perpendicular to the surface. Therefore, the depth of penetration may be less than in the case of KH204.

Based on results from earlier experiments carried out with phosphatidylserine (PS) monolayers, the ability of the phenylpropylamines to displace calcium bound to the monolayer had been reported (Hauser et al., 1969; Lullmann et al., 1980; Lullmann and Vollmer, 1982; Tabeteh, 1999; Klein et al., 2001). Hydrophobicity was shown to be an important factor in the binding of cationic amphiphilic drugs to phospholipids. Results from some of these earlier experiments showed the substance KH241 had the greatest ability to displace bound calcium from the phosphatidylserine monolayer. From figure 4-3, it is also clear that the substance has a log P value higher than that of KH204. But KH204 induces a greater reduction in T_t than KH241.

From figures 4-2 and 4-3, it is evident that the correlation between lipophilicity and T_t reduction using DPPA is a rather weak one. Further examples substantiate the difference that is evident between KH241 and KH204. The substances KH213 and CK19 have log P values that are more than a log value apart. Yet they have the same ΔT_t values. On the contrary, although the log P values of CK92 and KH204 do not lie far apart, their ΔT_t values do.

4.5.4 Derivatives with a methoxy-residue and the significance of compound length

The two compounds KH216 and CK41 possess methoxy-residues. The ΔT_t brought about by the KH216 and CK41 are essentially similar, indicating that the additional phenyl group attached to the propylchain does not influence the effect. The likely explanation for this is that both agents have the same depth of penetration into the DPPA-bilayers. The same applies to KH216 compared with KH210.
The structurally similar CK84 that lacks the methoxy-substituent compared with CK41 reduces T_t much less. Again, the role played by the length of the compound becomes evident, for the presence of the methoxy-substituent accounts for the difference in activity observed between CK41 and CK84.

Despite their obvious structural differences, the substances KH213, CK19, CK92 and KH220 bring about a similar reduction in T_t as can be seen on figure 4-1. KH220, with its naphthalene substituent is not capable of decreasing T_t any more than the much simpler KH213 and CK19. This probably lies in the planar nature of naphthalene. The presence of the second benzene ring in the meta-position relative to the propyl chain in CK92 reduces the entire length of the molecule considering the propyl chain as reference axis perpendicular to the surface. The ΔT_t of these substances do not differ significantly from each other, the only exception being the ΔT_t brought about by CK92 compared with KH220 with a slight significant difference (p just below 0.05)
Thus for structurally similar substances, the difference in ΔT_t is brought about by differences in the lengths of the compounds involved. The lipophilicity may facilitate the ease with which a substance penetrates and resides within the phospholipid bilayer but ultimately, the depth of penetration and the spatial arrangement of the substance seem to be crucial in determining the induced ΔT_t.
The minimal deviation of the values from the means evident in the bars demonstrates the existence and clear-cut nature of the above-described domains.

4.5.5 Effects of allosteric modulators

The substance W84 is a typical allosteric agent in muscarinic M_2 receptors, exercising a negative cooperative interaction with the binding of the orthosteric agent tritium N-methylscopolamine, [^{3}H]NMS to M_2 receptors. The cooperativity is a measure of the mutual influence on the affinity to the respective binding sites between the orthosteric

agent and the allosteric modulator. Replacing one of the quaternary nitrogen atoms in this bisammonium molecule, W84, with a silicon atom and varying the length of the central alkyl chain switched negative to positive cooperativity in most cases, leading to the Si-containing compounds being enhancers of [^{3}H]NMS binding.

While carbon and nitrogen are members of the second period of the periodic table, silicon is one period lower. This means the silicon atom is considerably larger, more so when compared with the positively charged nitrogen. Consequently, a silicon-containing derivative with a shorter alkyl central chain than that of W84 was required in order to allow a direct comparison with W84. The substance TD5 came closest to fulfilling this prerequisite. This substance exhibited positive cooperativity as opposed to W84, leading to an augmentation of [^{3}H]NMS binding.

Tests on T_t were carried out with both substances, W84 and TD5. The presence of two positive charges in W84 did affect the phospholipids differently from that of just one (Figs. 3-21 and 3-22). While the compound TD5 produced signals that varied in form and size over the entire measured range, the signals produced by the influence of W84 remained largely unchanged. It is likely the neutral silicon-containing moiety penetrates deep into the phospholipid bilayer. As mentioned in section 4.2, it has been proposed by Hanpft and Mohr (1985) that the interaction of positively charged test substances with the DPPA molecules annuls the specificity brought about by the negative charge of the DPPA headgroups. Assuming no further interaction, T_t is reduced to that of pure DPPC liposomes. In the case of TD5 in DPPA liposomes, the peak appears well below the main T_t of DPPC and it assumes a different shape at a molar ratio of 1.0. With a maximum ΔT_t value of about 30°C, the substance penetrates the bilayer to an extent that is at least similar to that of the substance KH211. In the case of W84, the major transition peak remains small, and the T_t value stays just at about the T_t value of pure DPPC. It seems plausible to assume that the substance stays predominantly on the surface in the area of the head groups and neutralises the head group charge.

The substance naphmethonium was a result of efforts by Holzgrabe et al. (2000) to find high affinity modulators to the [^{3}H]NMS occupied M_2 receptors beginning with W84. Furthermore, naphmethonium is an enhancer of NMS-binding to M_2 receptors. In tests with DPPA liposomes, the substance initially produced a multi-peak substance-induced signal before this latter eventually became a single peak (Fig. 3-25). The difference in structure of the compound compared with W84 is the replacement of one of the phthalimidopropyl residues in W84 by the more voluminous naphthylimido-ß-

dimethylpropyl residue. This induced a significant difference in the T_t. The slightly higher ΔT_t from naphmethonium can thus be attributed to the larger size of the naphthalimide rest. The final main peak is not only larger than that induced by W84, the T_t value is also lower than that of W84. By all indications, at least a part of the substance penetrates the phospholipid bilayer more than W84 does. The multi-peak signal also means the substance interacts with the phospholipid molecules in a more complex manner.

The substance Duo3 induced an initial reduction in T_t, with a ΔT_t of about 13°C up to a molar ratio of 0.2 (Fig. 3-21). T_t eventually increased above a molar ratio of 0.5 with a ΔT_t of about 5°C (Fig. 3-23). Wduo3 acted similarly but the absolute reduction and increase in T_t was smaller. The peak shapes were also different. Wduo3 induced a multi-peak signal at a molar ratio of 0.2, with such a signal lacking in the Duo3 thermograms. The minimum T_t value obtained using Duo3 lay by 50°C, Duo3 being the only substance that could apparently not annul the DPPA head group influence, and so could not attain a T_t value close to 41°C. This behaviour is interesting, since Duo3 was one of the substances described as an atypical allosteric modulator (Traenkle et al., 2003). The mode of interaction of this substance with the liposomes seems to differ from that of the substance W84.

Wduo3 also produced signals with peaks higher than the T_t of pure DPPA, but at least at a certain concentration, the substance-induced peak was less than that of pure DPPC. Thus, the head group specific effect of DPPA was annulled.

4.6 Effects on DPPC liposomes

As explained earlier, the signals resulting from the interaction of test substance with DPPC phospholipids in liposomes display a gradual change in T_t with increasing amounts of test substance. This change, usually a reduction, approaches a threshold value at a certain molar ratio of substance to phospholipid. The concentration at which this occurs is substance specific and depends on the mode of interaction of the substance with the phospholipid. With some of the test substances, this value is reached at the relatively low molar ratio of 0.5, while with others, this saturation value is not reached even at a molar ratio of 3.0. In cases where the maximum molar ratio measured was below 2.0, the limiting factor was usually the emergence of a detergent-like effect of the substance, that has been described in previous works (Hanpft and Mohr, 1985; Hanpft, 1987). At this molar ratio and beyond, the dispersion became clear and colourless and the consistence changed with the viscosity dropping.

4.6.1 Effects of propranolol on DPPC liposomes

The influence of propranolol on the T_t of DPPC was measured to a mole ratio of 3.0. At this value, the dose effect curve had attained a plateau with a total reduction in T_t of 11°C.

Jutila et al. (1998) found that the fluidity of the membranes also changed with the mole fractions. When the mole fraction of propranolol to phosphatidylcholine was 0.20, propranolol decreased membrane fluidity, while at a molar ratio of 1.00, propranolol rigidified membranes. This condition was reflected in this work, with a fluid milk-white dispersion observed at a mole fraction of 0.2 and a viscous, jelly-like emulsion at a molar ratio of 1.0. At higher molar ratios, the viscosity decreased. Figure 3-29 also shows the characteristic shape of the curves that resulted from measurements with a molar ratio above 2.0. Thus, not only did the consistence of the resulting emulsions change but also the shapes of the curves.

4.6.2 Effects of the phenylpropylamines on DPPC liposomes

Figures 4-4 and 4-5 show summaries of the effect of the various phenylpropylamines on the T_t of DPPC liposomes. Figure 4-4 contains substance-effect curves from substances with an alkyl substituent in the para-position of the phenyl ring. As is evident on the graph, the molar ratio required to attain saturation varied considerably

among the individual substances. The peak shapes produced by these substances also changed with molar ratio. The initially narrow and symmetrical peaks generally got broader and asymmetrical before becoming narrow and symmetrical again with the exact molar ratios at which the differences were observed depending on the substance. Also, of all the substances tested, liposome dispersions with the highest molar ratios could be produced with these simple phenylpropylamines without the ensuing of the detergent effect.

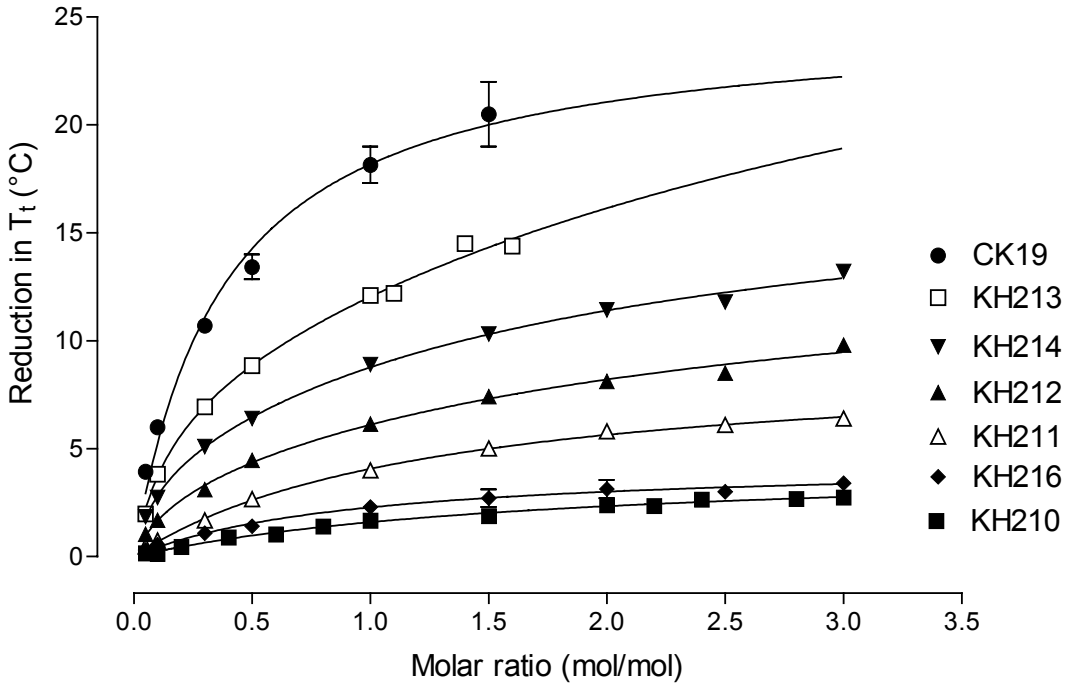


Figure 4-4: Substance-effect curves of phenylpropylamines with a simple substituent in para position. The curves show the substance-induced ΔT_t obtained from calorimetric measurements performed using DPPC liposomes containing the indicated test substances in the corresponding molar ratios. ***Ordinate***: reduction in phase transition temperature. ***Abscissa***: molar ratio of test substance to phospholipid.

The curves of many of the phenylpropylamines had not attained a plateau level at the maximum measured molar ratio. However, those that did attain a plateau level did not bring about a reduction similar to that brought about using DPPA (compare plateau values with those in table 3-17).

4.6.3 Compounds with further phenyl groups

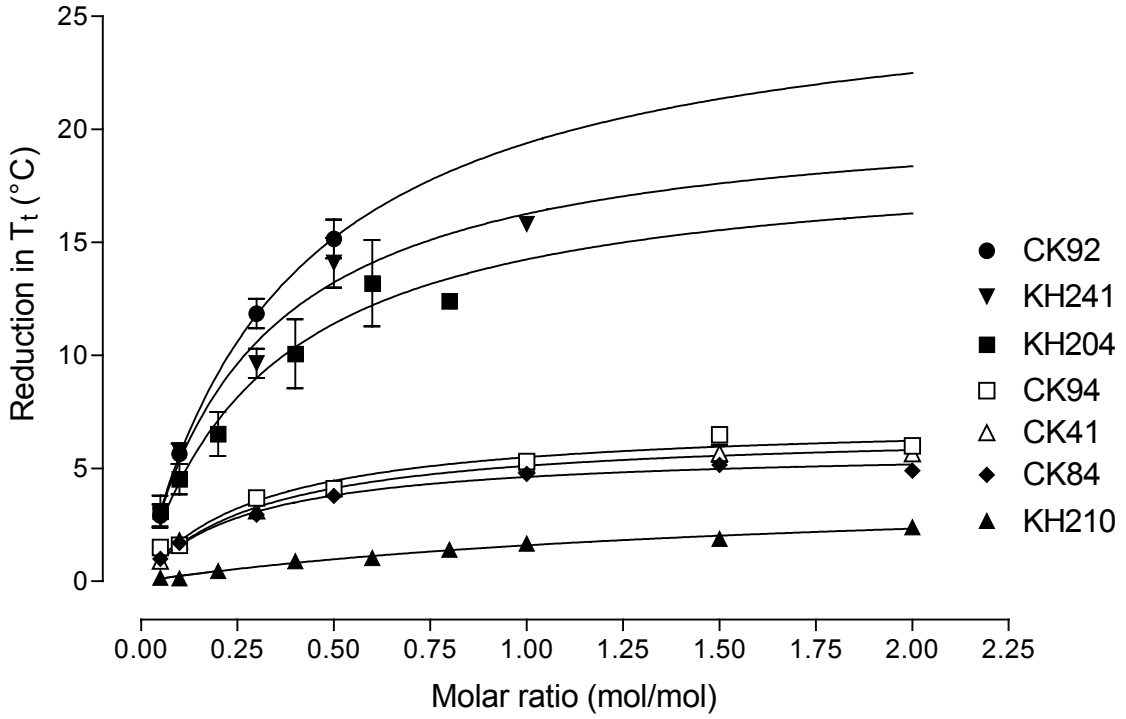


Figure 4-5: Substance-effect curves of bi- and diphenylpropylamines with that of KH210 shown for comparison. The curves show the substance-induced ΔT_t obtained from calorimetric measurements performed using DPPC liposomes containing the indicated test substances in the corresponding molar ratios.
Ordinate: reduction in phase transition temperature. ***Abscissa***: molar ratio of test substance to phospholipid

It is evident from the thermograms in section 3.5 that the determination of the exact onset values of the peaks resulting from the interaction with biphenyl substances (CK92, KH204, KH241) was generally more difficult due to the shapes of the peaks that resulted in many molar ratios. A saturation point could not be easily ascertained. The diphenyl substances (those having both phenyl rings attached directly to the propyl chain) generally attained saturation at very high T_t values, meaning a small reduction in T_t.

With DPPC, the upper limit of the applied molar ratio measured was usually a result of the ensuing of the already mentioned detergent effect. Here, the dispersion became a clear liquid or there was a separation in a clear liquid phase and a solid phase that was translucent in nature. This was particularly the case with the biphenyl compounds, that is, substances with adjoining phenyl groups. While it was hardly possible to measure beyond or even attain a molar ratio of 1.0 with these compounds, much higher molar ratios could be applied with the other simple phenylpropylamines and also with the

diphenyl substances. The maximum applicable molar ratios were not limited solely by the detergent effect. Particularly with DPPA, this was restricted by different factors: at higher molar ratios especially when the dispersions were cooled to room temperature they acquired the consistence of an amorphous solid, making it difficult to pipette a sample into the aluminium pans. These substances could hardly be applied beyond a relatively low molar ratio of substance to phospholipid, mostly 1.0.

4.6.4 Effects of the muscarinic acetylcholine modulators on DPPC liposomes

While the substance W84 did not have any detectable effect on the T_t of DPPC under the experimental conditions, its silicon-containing analogue, TD5 did (Fig. 3-61).
Whereas the bisammonium compound W84 does not seem to penetrate the DPPC bilayer, the contrary could be assumed for its silicon analogue (Table 4-1). This may be relevant to the manner in which these substances react with lipophilic domains of membrane proteins especially receptor proteins, further strengthening the findings of Duda-Johner (2002).
The different shapes of the signals in figure 3-61 are difficult to explain. Reducing the interaction to an intercalation of the TD5 molecules into the phospholipid bilayer alone would hardly be satisfactory. It is likely, that the TD5 and the phospholipid molecules influence each other mutually.
The substance naphmethonium did not seem to have a similar effect on the T_t of DPPC. Despite the presence of the much larger naphthylimido-ß-dimethylpropyl residue, the substance only brought about a minimal reduction in T_t, and although the mean T_t values measured to a mole ratio of 1.5 differed significantly from that of pure DPPC ($p<0.05$), the difference in activity compared with W84 is hardly evident. This means if the substance interacts with the DPPC phospholipid bilayer differently from W84, this difference is minimal. This contrasts sharply with the results obtained with DPPA phospholipids where the differences in the peak shapes of the two substances indicated a clear difference in the manner of the interaction of the two substances with the phospholipid molecules.
While the substance Duo3 had a clearly noticeable reducing effect on the T_t of DPPC (Fig. 3-64), an effect induced by Wduo3 was not so conspicuous (Fig. 3-66). This difference is probably coupled with the lipophilicity of the substituents involved, their lengths playing a minor role. The signals produced by Wduo3 at molar ratios beyond

0.1 did not only contain peaks that were asymmetrical, but the peaks appeared to be merged multiple peaks, an indication of the possible existence of domains with different compositions. The reduction in T_t brought about by Duo3 amounted to an average of 3.5°C with the signals obtained at higher molar ratios being relatively constant in shape and size.

Below is a summary table with values obtained from the compound W84 and related substances on the T_t of DPPC.

Substance	Mean drug-induced T_t (°C)	$\Delta T_t \pm$ SEM (°C)	n
W84	42.9 ± 0.10	0.0 ± 0.06	3
Naphmethonium	42.6 (± 0.05)	0.2 (± 0.10)	2
Wduo3	40.1 (± 0.18)	1.3 (± 0.35)	2
Duo3	37.1 ± 0.42	3.5 ± 0.17	3
TD5	33.9 ± 0.10	8.5 ± 0.32	3

Table 4-1: Summary table with statistical data on the phase transition temperatures resulting from experiments carried out with W84 and related substances at a molar ratio of unity between substance and DPPC. SEM: standard error of mean. n: number of experiments considered. Some seven to eight values resulted from each experiment, depending on the number of molar ratios measured.

As mentioned in the introduction, typical allosteric modulators are ligands that bind normally at the common allosteric site. Antagonists binding to this very site can displace these typical modulators (Ellis and Seidenberg, 1992). The substances W84 and Wduo3 could be identified as ligands at the common allosteric site in binding studies, while this was not the case with the substance Duo3 (Trankle and Mohr, 1997).

Schröter (1999) performed experiments with Duo3 on [^{3}H]-NMS occupied and free M_2-receptors to determine if the interaction with receptors in these two states were similar. The findings suggested that these were similar and the substance binds in the same domain in both cases. However, the study was not designed to find out whether other binding sites but the common allosteric binding site were involved in the action of Duo3. Leppik et al., and other groups postulated the existence of an additional allosteric binding site. After all, a binding site for cations had been found that modulates ligand binding in muscarinic receptors (Rosenberger et al., 1980; Gerstin et al., 1992; Leppik et al., 1994). Further experiments with more muscarinic receptor subtypes (M_1-M_4) suggested the existence of at least one additional allosteric binding

site (Lazareno et al., 2000; Birdsall et al., 2001) that the authors termed a “second allosteric site”.

Experiments conducted for Duo3 with chimeric receptors did indeed reveal the existence of a binding domain that is topologically different from the common allosteric site (Dittmann, 2003). Despite the existence of this second site, the atypical concentration-effect curves of certain substances such as Duo3 still remain unexplained.

Mieskes (1999) showed that modulators of great structural diversity can bind unto phospholipids, but the concentrations in which these effects occur were not directly related to the allosteric effect of modulators. Nevertheless, it is intriguing that the typical allosteric agents W84, naphmethonium, and Wduo3 have no or only a marginal effect on the phase transition behaviour of DPPC whereas the atypical compounds Duo3 and TD5 induce a clear reduction of the main transition temperature. This finding suggests that the latter compounds have the propensity to penetrate into a hydrophilic/hydrophobic interphase.

With respect to the muscarinic M_2 receptor, this could mean that the compounds do not only attach superficially to the receptor protein but that parts of the molecule intercalate in hydrophobic areas of the receptor or even reach the surrounding phospholipids.

It is tempting to speculate that the atypical features of the allosteric action are sometimes based on the propensity of these compounds to penetrate into hydrophilic/hydrophobic interphases.

4.6.5 General remarks

While an interaction with the prototype modulator W84 was only evident in DPPA liposomes under the experimental conditions, effects were evident with the atypical modulators TD5 and Duo3 using both phospholipids under similar conditions. From considerations of the thermograms resulting from the phenylpropylamines KH204 and the more voluminous KH241, it can be deduced that T_t is not only influenced by the depth of penetration into the bilayer (which in itself depends on the lipophilicity of the substance), but also by the spatial arrangement of the immersed molecule moiety. Thus in addition to the chemical interactions involved, strong physical forces seem to influence these latter as well. The simpler KH204 brought about a greater reduction in T_t than the more voluminous KH241. It is conceivable that the various parts of the molecule squeeze the phospholipid acyl chains closer together, increasing their

abilities to form van der Waals interactions with one another and so rigidifying them. The physical process thus enhances the chemical processes involved. The measured resultant is a product of these effects, among others and in this case, this leads to a ΔT_t that is less than that brought about by the simpler KH204. With TD5, a neutralisation of the head groups as well as a penetration into the hydrocarbon chains is clearly evident. In the case of DPPA, the phospholipid head groups would be neutralised by the positively charged quaternary nitrogen and the neutral rest of the molecule penetrates into the bilayer. In the case of DPPC, there is a reduction in T_t caused by the penetration of the neutral moiety into the hydrocarbon chain. Despite the penetrating moiety of TD5 being much longer, the ΔT_t brought about by TD5 is less than that caused by KH204, probably due to the above mentioned rigidifying effect of the silicon-containing voluminous and yet compact three-dimensional structure of TD5 squeezing the hydrocarbon chains together as opposed to the planar nature of the biphenyl group of KH204.

The thermograms from DPPA liposomes containing Wduo3 and Duo3 could result from a similar scenario. However, the ability to form loose attractive forces between the immersed moiety and the hydrocarbon chains would also contribute in the final outcome.

From a further look at the results of the other allosteric modulators using DPPA, it can be deduced that the neutralisation of the headgroups is accompanied by a change in orientation of the phospholipid molecules, prompting a limited interaction between certain polar molecules with the acyl chains, a fact that can be seen in the signals resulting from tests with naphmethonium (section 3.2.3.5). This led to a slight reduction beyond that obtained using pure DPPC. With the phospholipid, DPPC, which is neutral under the experimental conditions, such a reduction was absent.

5. Summary

The essential biomembrane components, phospholipids, interact with various drug substances among others. The physicochemical properties of the phospholipids are not only a prerequisite for their formation of bilayers; these properties also enhance interactions with amphiphilic substances.

Ligand binding to muscarinic acetylcholine receptors can be modulated by various substances. These allosteric modulators are classified as typical and atypical on the basis of radioligand binding experiments.

We aimed at using phospholipid bilayers to study whether selected typical and atypical modulators differ in their ability to interact with hydrophobic/lipophilic interphases. We used the method of differential scanning calorimetry (DSC) and measured drug effects on the phase transition temperature, T_t, of modulator-containing liposome suspensions compared with the suspension of pure phospholipids. The typical modulators used were Wduo3 (1,1'-(1,3-propandiyl)-bis[4,4'-phthalimidomethoxyl-iminomethyl-pyridinium]-dibromide), W84 (hexane-1,6-bis(dimethyl-3´-phthalimidopropyl-ammonium) dibromide) and its derivative naphmethonium (a phthalimidopropyl residue in W84 is replaced by the more voluminous naphthylimido-ß-dimethylpropyl residue). The atypical modulators were TD5 (a silicon-containing derivative of W84 with a quaternary ammonium nitrogen replaced by silicon) and Duo3 (1,1'-(1,3-propandiyl)-bis[4,4'-(2,6-dichlorbenzoxyl)-iminomethyl-pyridinium]-dibromide).

The phospholipids used were dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidic acid (DPPA). The buffer, a 14mM TES/14mM histidine buffer adjusted with HCl to pH 6 [TES = *N*-tris (hydroxymethyl)-2-aminoethane-sulfonic acid] ensured that DPPA was ionic while DPPC was neutral under the experimental conditions. That way, the role played by the difference in headgroups could be examined. The average T_t values of pure phospholipid liposome suspensions were 41.5 ± 0.21°C ($\bar{x}$ ± SEM, n = 30) and 63.4 ± 0.15°C ($\bar{x}$ ± SEM, n = 35) for DPPC and DPPA, respectively.

Measurements were first carried out using a set of systematically varied phenylpropylamines to establish structure-activity relationships. The findings suggested that the drug-induced T_t depends to a great extent on the depth of penetration into the phospholipid bilayer and also on the structure of the penetrating lipophilic moiety.

On the basis of these findings the results of experiments with the allosteric modulators were interpreted. The typical modulator W84 induced a reduction of T_t in DPPA liposomes to the T_t value of pure DPPC. The substance thus eliminated only the head-group specificity of DPPA and does not seem to penetrate into the bilayer. This was confirmed by the absence of an effect by W84 on the T_t when measured with DPPC liposomes (ΔT_t = 0,0 ± 0.06°C [$\bar{x}$ ± SEM, n = 3]). The substance naphmethonium acted similar to W84, though the presence of the larger lipophilic substituent caused a reduction in T_t of DPPA to a value slightly lower than that of pure DPPC (T_t = 38.5 ± 0.11°C [$\bar{x}$ ± SEM, n = 5]). The substance had a marginal effect on the T_t of DPPC liposomes (ΔT_t = 0,2 ± 0.10°C [$\bar{x}$ ± SEM, n = 2 values at molar ratio of 1.0]).

The effect of the silicon-containing atypical modulator TD5 on DPPA liposomes was a reduction that was molar ratio-dependent, contrary to the uniform molar ratio-independent values obtained from W84 and naphmethonium experiments with DPPA. At the substance to DPPA molar ratio of 1.0, TD5 produced a signal with a peak seemingly resulting from the superimposition of two peaks with a T_t value lower than that of pure DPPC (T_t = 33.8 ± 0.20°C [$\bar{x}$ ± SEM, n = 3 values at molar ratio of 1.0 from 3 experiments]). This was the greatest reduction measured among the allosteric modulators. A reduction in T_t of DPPC to the value obtained from the DPPA experiment was also achieved (T_t = 33.9 ± 0.10°C [$\bar{x}$ ± SEM, n = 3]). TD5 was the only substance that reduced the T_t of both DPPA and DPPC liposomes to approximately the same level at a molar ratio of 1.0.

The effect of the atypical bispyridinium-type modulator Duo3 on the T_t of DPPA was complex; an initial reduction was followed beyond a molar ratio of 0.5 by an increase in T_t above that of pure DPPA. Besides, this was the only compound that did not reduce the T_t of DPPA to a value as low as that of pure DPPC. But the substance did reduce the value of DPPC (T_t = 37.1 ± 0.42°C [$\bar{x}$ ± SEM, n = 3]). While W84 had no effect on the T_t of DPPC and the bispyridinium-type modulator Wduo3, which is a typically acting allosteric agent only had a marginal effect (T_t = 40.1 ± 0.18°C [$\bar{x}$ ± SEM, n = 2]), Duo3 did induce a clearly significant reduction in the T_t of DPPC (T_t = 37.1 ± 0.42°C [$\bar{x}$ ± SEM, n = 3]).

Taken together, the finding reveal that typical and atypical modulators differ in their ability to interact with hydrophilic/lipophilic phospholipid interphases. It is tempting to suggest that the molecular mode of interaction with hydrophilic/lipophilic interphases of

the muscarinic receptor protein is likewise different between the compounds and that this difference may be involved in atypical versus typical allosteric actions.

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7. Appendix

Amphotericin B

The substance amphotericin B acts through an interaction with membrane components, forming pores that contain hydrophilic interiors in the membranes, leading to a loss of membrane-selective permeability and accompanied by an eventual loss of cytoplasmic components.

Being amphiphilic, experiments were also carried out in this work using the compound. Due to the photosensitive nature of amphotericin B, the experimental setting was designed to ensure the vessels were shielded from light at all times.

The results of one of such experiments are shown in the thermograms in the figure below.

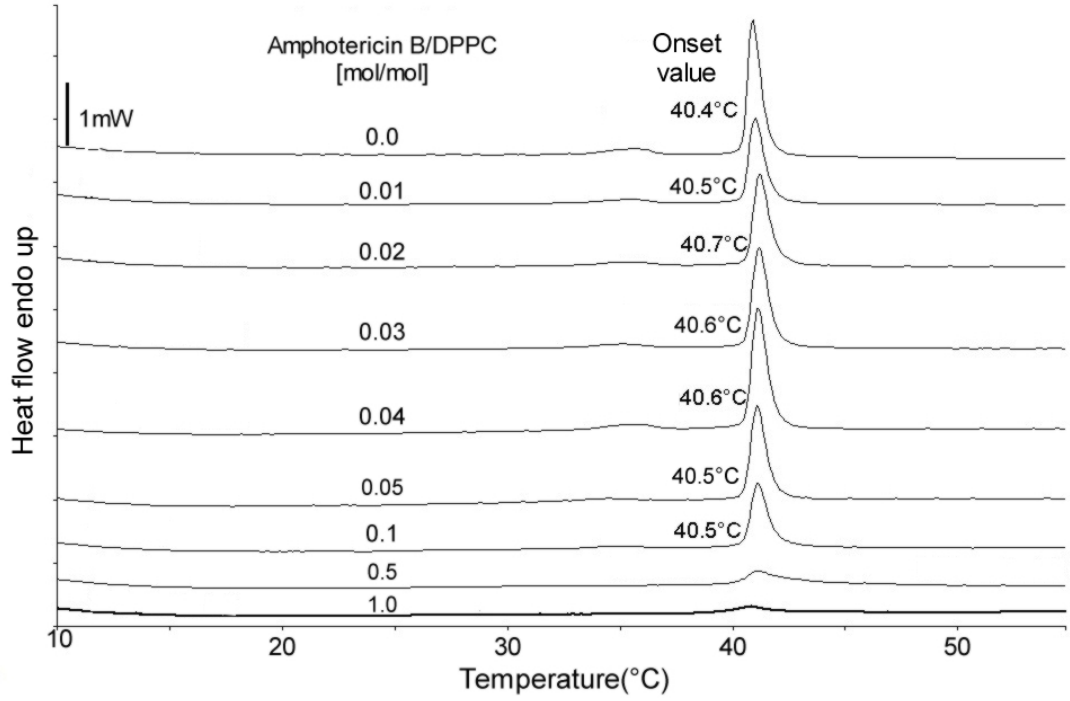


Figure 7-1: Original thermograms obtained from DSC measurements, using DPPC-liposome dispersions containing the substance amphotericin B in the indicated molar ratios. This is representative of 3 experiments. A statistical test of the values from all three experiments did not show a significant difference in T_t values obtained from the various molar ratios compared to the values using pure DPPC. ***Ordinate***: endothermic heat flow. ***Abscissa***: sample temperature in °C.

As can be seen from the thermograms in the figure, hardly any extra transition peaks other than the pre- and the main transition peaks from DPPC are present.

Fournier et al. (1998) also carried out calorimetric experiments with amphotericin B to investigate the latter's effects on pure and ergosterol- or cholesterol-containing DPPC. Their results showed an interaction of amphotericin B with pure phospholipids, with thermograms containing peaks at temperatures higher than that of pure DPPC.

The pH value of 6.0 used in this work was so chosen to maintain consistency with the conditions used in investigating the other test substances. While a TES-histidine buffer was used here, Fournier et al. used a phosphate buffer solution with a pH value of 7.0. No further experiments were carried out with regards to pH since this was not the objective of the present work. Also, further details concerning the various measuring parameters used by Fournier et al. could not be found in the literature.

8. Structural Formulae

Tables containing structural formulae of investigated substances

Phenylpropylamines I

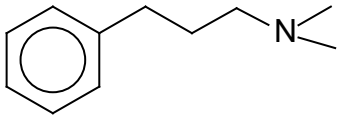
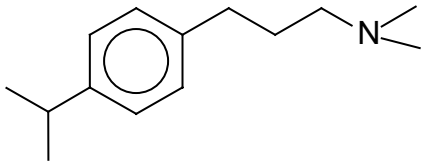
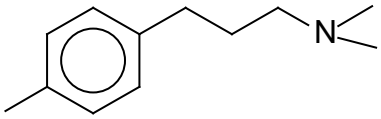
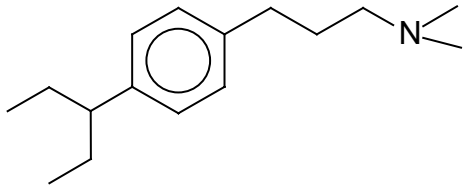
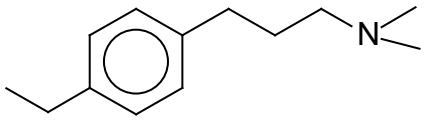
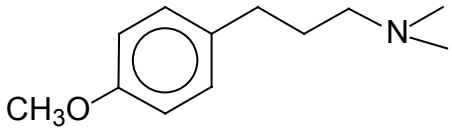
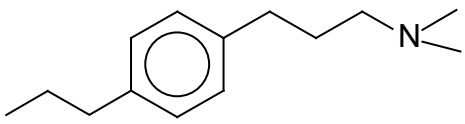
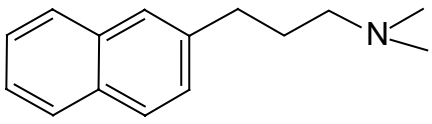
Test Substance	Test Substance
KH210 	KH214 
KH211 	CK19 
KH212 	KH216 
KH213 	KH220 

Table 8-1: Shown here are the structures of the simple phenylpropylamines. A general idea of the lengths of the molecules can be obtained from the depicted structural formulae.

Phenylpropylamines II

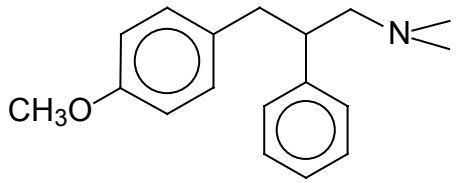
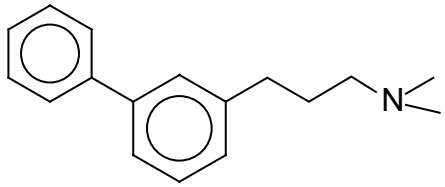
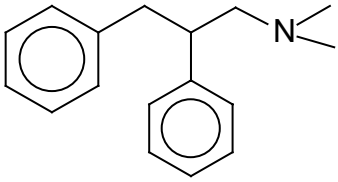
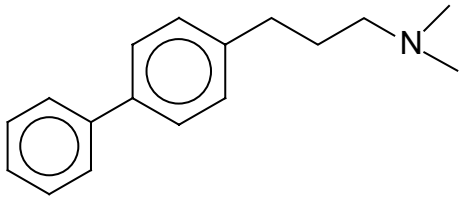
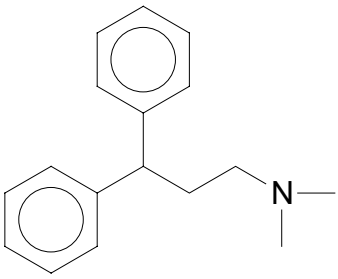
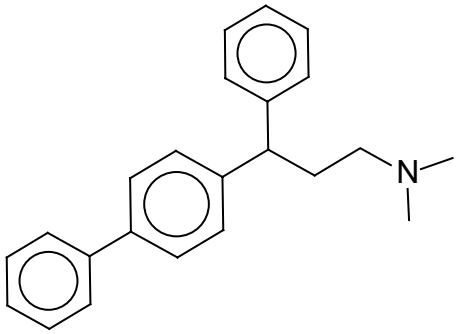
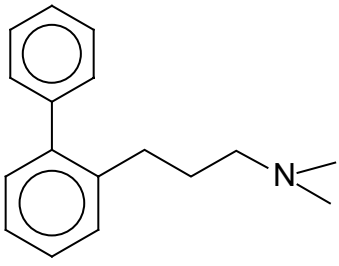
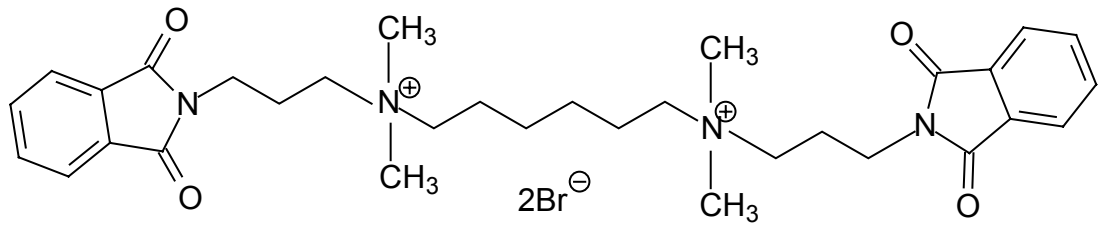
Test substance	Test substance
CK41 	CK92 
CK84 	KH204 
CK94 	KH241 
CK53 	

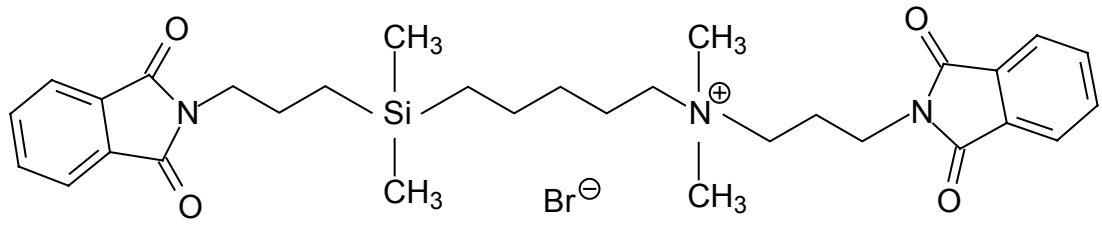
Table 8-2: Table containing di- and tri-phenylpropylamines.

W84 and further allosteric modulators

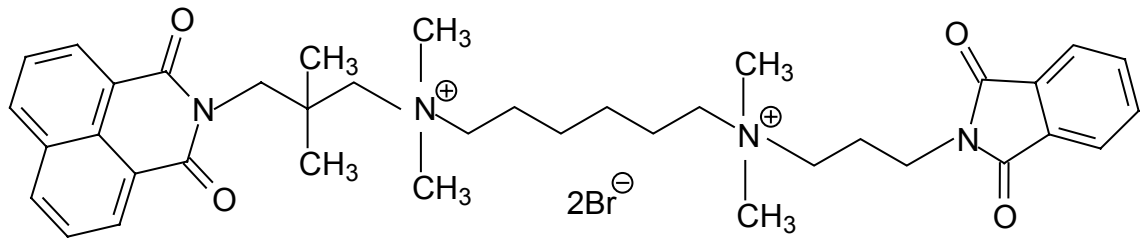
W84



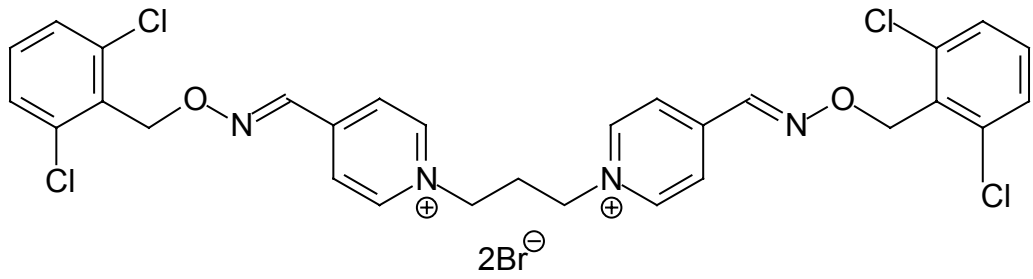
TD5



Naphmethonium



Duo3



Wduo3

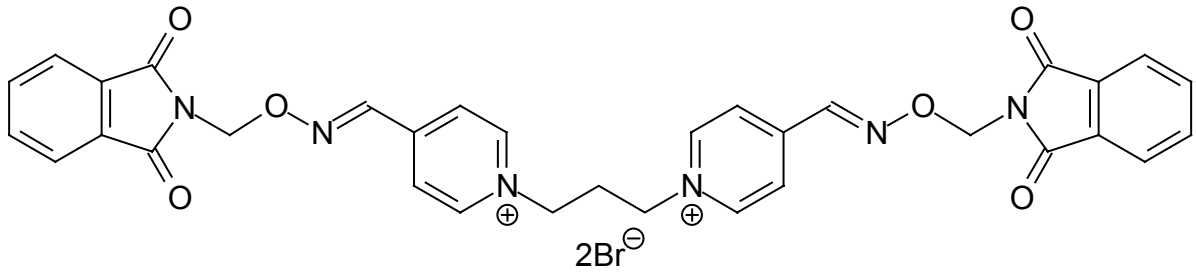


Table 8-3: Structural formulae of the M_2-receptor allosteric modulator W84, its silicon derivative and further modulators.

9. Abstracts

Frunjang, G. T., Muth, M, Holzgrabe, U., Daiss, J. O., Tacke, R., and Mohr, K. (2003) Probing the interaction of muscarinic allosteric agents with a model interphase. *Naunyn Schmiedebergs Arch. Pharmacol.* **367** (Suppl.): R26, 92.