Disruption of Phospholipid Packing by Branched Poly(ethylenimine) Derivatives†

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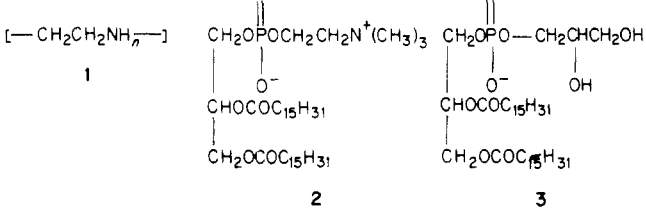
ABSTRACT: High-sensitivity differential scanning calorimetry was used to examine the phase transition behavior of mixtures of dipalmitoylphosphatidylcholine (DPPC) or dipalmitoylphosphatidylglycerol (DPPG) with linear or branched poly(ethylenimine) derivatives. Unmodified poly(ethylenimines), branched or linear, caused no observable change in the melting of DPPC. Both polymers broadened the DPPG melting endotherm and produced small changes in the melting temperature. Attachment of side chains 6–12 carbon atoms in length produced hydrophobic poly(ethylenimine) derivatives which bound strongly to DPPC with complete disruption of the multilamellar vesicle structure.

Introduction

The factors which control the interactions of synthetic polymers with natural or synthetic bilayer membranes, and the structural consequences of those interactions, are poorly understood. We believe that an understanding of polymer–bilayer interactions will allow significant advances in biomaterials development and in the use of phospholipid and surfactant vesicles in the control of chemical and biological processes. This paper describes a part of our ongoing study of polymer–bilayer interactions via chemical,[1] thermodynamic,[2-9] and spectroscopic[9] methods. In particular, we discuss the use of high-sensitivity differential scanning calorimetry to examine the interaction of poly(ethylenimine) derivatives (1) with bilayers prepared from the synthetic phospholipids dipalmitoylphosphatidylcholine (2) and dipalmitoylphosphatidylglycerol (3).



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Similar studies of other polymer–lipid mixtures have revealed a remarkable diversity in the nature of polymer–bilayer interactions. In the preceding paper in this series,[2] we described a pH-dependent complexation of poly(carboxylic acids) with phospholipid vesicle membranes, the consequences of which varied from a rather minor loss of cooperativity in the thermal phase transition of the bilayer at high pH to the complete disappearance of large lipid vesicles at low pH. In earlier work on the interactions of ionene-type polycations with dipalmitoylphosphatidylglycerol,[7-9] we found that adsorption of these linear polycations on the bilayer surface results in an increased ordering of the low-temperature lipid phase, and a competitive displacement of surface-bound divalent metal cations.[9] In this paper, we discuss the disruption of bilayer structure which occurs upon treatment of lipid suspensions with branched poly(ethylenimine) derivatives.

Experimental Section

Materials. Branched poly(ethylenimine) (PEI_b) with a stated molecular weight between 40 000 and 60 000 was purchased from Polysciences, Inc., as a 33% aqueous solution. Dipalmitoyl-L-α-phosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids, Inc. Dipalmitoyl-L-α-phosphatidyl-DL-glycerol, ammonium salt (DPPG), and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were purchased from Sigma Chemical Co. All of the above materials were used without purification. High purity of the lipid samples was confirmed by thin-layer chromatography and by the sharpness of the thermal phase transition (peak width at half-maximum 0.13 °C for DPPC and 0.35 °C for DPPG). Methyl hexanoate (Pfaltz and Bauer) and methyl laurate (Aldrich) were distilled before use. Dialyses were performed with Spectrapor membrane tubing (Spectrum Medical Industries) with a molecular weight cutoff of 1000.

Preparation of Modified PEI_b's. Branched poly(ethylenimine) derivatives bearing pendent hexanoyl and lauroyl chains were prepared according to Klotz et al.[10] Water from the aqueous solution of PEI_b was removed in the rotary evaporator. The residue was dissolved in dry ethanol and the solvent removed; after repetition of this sequence, the polymer was dissolved in dry ethanol to produce a 35% (w/v) solution. The desired methyl ester was added, and the solution heated to reflux, with stirring, for 72 h. The mixture was diluted with ethanol to 3 times its original volume, and the polymer was precipitated by bubbling anhydrous HCl through the solution. The polymer was washed extensively with ethanol and dried under vacuum for several days at 45 °C. The extent of acylation was determined by 300-MHz ^1H nuclear magnetic resonance spectrometry. All polymer solutions were dialyzed against 20 times their volume of 50 mM Tris-HCl buffer with mild stirring for 16 h, with one change of buffer, prior to use in calorimetry.

Preparation of PEI_l. Poly(2-ethyloxazoline) with a stated weight-average molecular weight of 50 000 was a gift of Dr. Thomas T. Chiu of Dow Chemical Co. The polymer (50 g) was dissolved in 750 mL of 20% aqueous H_2SO_4 and heated to reflux for 24 h. The reaction mixture was cooled and cautiously neutralized with 60 g of NaOH in 60 mL of H_2O. The resulting PEI_l was washed repeatedly with H_2O, until the wash was neutral. The sample was purified by three recrystallizations from water and dried in vacuo at 65 °C.

Measurements. ^1H NMR spectra were recorded at room temperature in D_2O (for PEI_b) or $CDCl_3$ (for PEI_l) on a Bruker WM-300 spectrometer. Electron micrographs were taken on a Philips EM 300 electron microscope. Calorimetric scans were recorded on a Microcal, Inc., MC-1 scanning microcalorimeter. A heating rate of 10 °C/h was used in all calorimetric experiments. Lipid suspensions were prepared at ca. 50 °C by vortex agitation of dry lipid with 50 mM Tris-HCl buffer which contained the polymer of interest. Lipid and polymer concentrations were each 1 mg/mL unless otherwise stated. The polymer–lipid suspensions were degassed and transferred to the calorimeter via calibrated syringe. Transition enthalpies were determined by supplying a precisely known current to the reference cell of the calorimeter. Calorimetric samples were subjected to repeated scans until consistent thermal behavior was observed. In general, successive scans provided endotherms of increased breadth, but the overall appearance of the endotherms, and the enthalpies obtained therefrom, were little changed from the first heating. Of the three thermal phase transitions which are known to occur in aqueous

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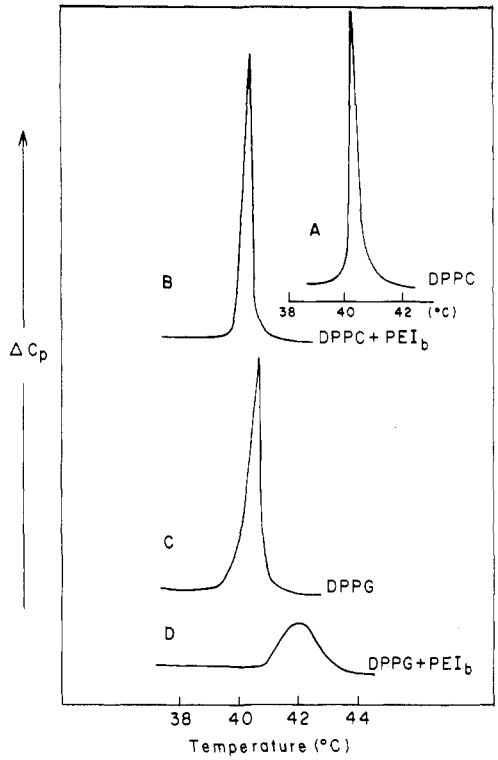

Figure 1. Main phase transition region for phospholipid dispersions (1 mg/mL) prepared by mechanical shaking in 50 mM Tris-HCl buffer, pH 7.4: (A) DPPC control, (B) DPPC plus 1 mg/mL PEI_b, (C) DPPG control, (D) DPPG plus 1 mg/mL PEI_b.

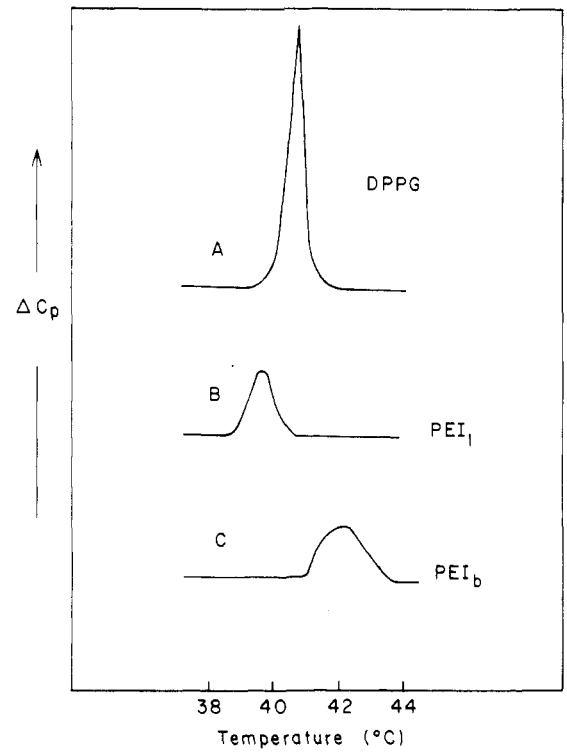

Figure 2. Main phase transition region for DPPG dispersions (1 mg/mL) prepared by mechanical shaking in 50 mM Tris-HCl buffer, pH 7.4: (A) DPPG control, (B) DPPG plus 1 mg/mL PEI_l, (C) DPPG plus 1 mg/mL PEI_b.

DPPC suspensions,[11] we discuss only the main transition. In general, the behavior of the pretransition was found to be similar to that of the main transition. In no sample did we observe a subtransition, nor did we attempt to do so.

Results and Discussion

It is well-known that hydration of either DPPC or DPPG in excess water at moderate pH and ionic strength produces a suspension of multilamellar vesicles which undergo a sharp order-to-disorder transition upon heating. For both of these synthetic phospholipids, this "main transition" temperature (T_m) is approximately 41 °C.[12] Figure 1 shows melting endotherms obtained on lipid suspensions prepared in dilute (0.1% w/v) aqueous solutions of branched poly(ethylenimine) (PEI_b) and demonstrates the expected critical role of lipid head-group structure in controlling the polymer–bilayer interaction. PEI_b under the conditions of these experiments (pH 7.4, 50 mM Tris-HCl buffer) leaves the DPPC melting endotherm essentially unchanged (curves A and B), but reduces the enthalpy, and increases the width and temperature, of the main transition of DPPG (curves C and D). Attractive electrostatic forces between the acidic DPPG surface and the basic PEI_b undoubtedly contribute to strong polymer–lipid binding in the latter system.

This modification of the ordered phase of DPPG by PEI_b contrasts sharply with our earlier results for mixtures of DPPG with ionene-type polycations.[7-9] Linear ionenes-3,3, -6,3, and -6,10 all interact strongly with DPPG under conditions similar to those of Figure 1, but the consequences of these interactions are quite different. The ionenes quite clearly stabilize the ordered phase of DPPG: ionene-treated DPPG suspensions exhibit elevated melting points and melting enthalpies, and extraordinarily narrow phase transitions. The magnitudes of these effects depend upon the charge density of the ionene, and become quite small for ionene-3,3. To the extent that poly(ethylenimine) may be considered a branched dealkylated ionene-2,2, the reduction in the size and cooperativity of the DPPG phase transition by PEI_b was unexpected.

Figure 2 illustrates the importance of chain branching in controlling PEI–DPPG interactions. Linear poly(ethylenimine) (PEI_l) *depresses* the DPPG T_m from 40.4 to 39.7 °C and broadens the transition slightly. This result may well reflect an extrapolation to higher charge density of the ionene-induced lipid modifications described above; at charge densities higher than that of ionene-3,3, linear polycations may in fact *destabilize* the ordered phase of acidic phospholipids such as DPPG because of a mismatch of optimal geometries for lipid packing and polymer–lipid ion pairing. Ion pairing requires a matching of the array of polymer-bound positive charges with the surface array of negatively charged lipid head groups. Such matching may be impossible in the unperturbed ordered lipid phase, if the separation of surface-bound anions (7.4 Å[13]) is greater than the maximum separation of successive positive sites on the polymer chain (3.8 Å[14]). Distortion of the bilayer in an attempt to optimize polymer–lipid ion pairing is thus suggested to account for the modified phase transition behavior shown in Figure 2. The origin of the chain branching effect is unknown, but it is interesting nonetheless to note the extent to which this structural parameter determines the consequences of the polymer–bilayer interaction.

Strong interactions between polymers and lipids bearing opposite charges are to be expected, and many parallel observations have been reported in structural studies of *protein*–lipid interactions. In an attempt to promote the interaction of PEI_b with the *zwitterionic* lipid DPPC, we prepared a number of PEI_b derivatives bearing apolar side chains. Figure 3 shows the melting endotherms obtained for DPPC suspensions prepared in 0.1% (w/v) solutions of PEI_b bearing either 20 (PEI_bH_{20}) or 50 (PEI_bH_{50}) mol % *n*-hexanoyl chains, or 15 mol % *n*-lauroyl chains (PEI_bL_{15}). As expected, the introduction of apolar side

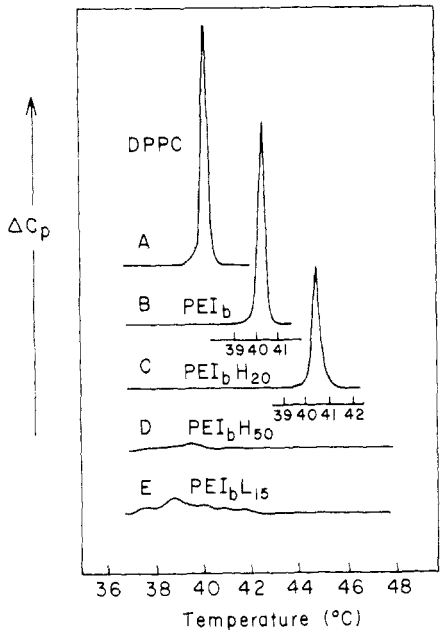

Figure 3. Main phase transition region for DPPC dispersions (1 mg/mL) prepared by mechanical shaking in 50 mM Tris-HCl buffer, pH 7.4: (A) DPPC control, (B–E) DPPC plus 1 mg/mL of modified PEI_b's as shown.

chains produces PEI derivatives which modify DPPC bilayer organization. While PEI_bH_{20} causes only minor broadening of the lipid phase transition, PEI_bH_{50} under the conditions of this experiment eliminates the endotherm entirely. The longer *n*-lauroyl side chain is more effective in disrupting bilayer organization; incorporation of only 15 mol % is sufficient to eliminate the phase transition. The loss of melting enthalpy is observed in these systems regardless of the method by which the polymer is introduced into the lipid suspensions: hydration of the lipid in the polymer solution or addition of the polymer to the preformed lipid suspension causes disappearance of the calorimetric phase transition. Small changes in solution pH (pH 6–9) and salt concentration (0.05–0.25 M) are also unimportant.

The correlation of the loss of melting enthalpy from the endotherms shown in Figure 3 with the level of incorporation of apolar side chains suggests hydrophobic association of the modified PEI_b's with the hydrocarbon core of the DPPC bilayer. Klotz has demonstrated hydrophobic binding of small organic molecules in aqueous solutions of modified PEI_b's[10,15] and reports similar sensitivity to side-chain length and concentration.

We sought additional insight into the nature of these polymer–lipid interactions through an examination of the relation of lipid phase transition enthalpy and polymer concentration. Figure 4 shows the results for PEI_bH_{50}. Significant loss of transition enthalpy is apparent at a polymer concentration of 0.1 mg/mL (at which the lipid is present in 10-fold excess by weight), and the melting endotherm becomes very broad and ill-defined at polymer concentrations ≥0.5 mg/mL. Figure 5 is a plot of ΔH_m vs. polymer concentration ([DPPC] = 1 mg/mL throughout) and demonstrates an apparently linear loss of transition enthalpy with increasing $[PEI_bH_{50}]$. Extrapolation of the best straight-line fit of the data in Figure 5 to $\Delta H_m = 0$ gives an *x*-axis intercept of 0.60 mg/mL.

These observations are similar to those made previously by other workers in studies of *protein*–lipid interactions. Reconstitution of hydrophobic proteins such as gramicidin,[16] glycophorin,[17] lipophilin,[18] $(Ca^{2+} + Mg^{2+})$-ATPase,[19,20] cytochrome b_5,[21] bacteriorhodopsin,[22] cardio-

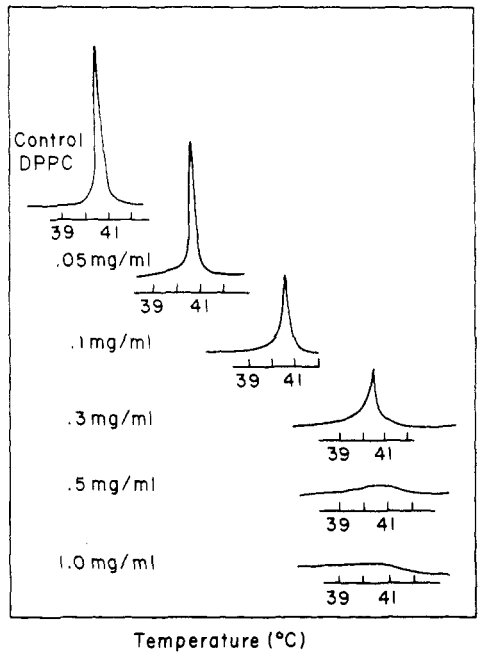

Figure 4. Main phase transition region for DPPC dispersions (1 mg/mL) prepared by mechanical shaking in 50 mM Tris-HCl buffer, pH 7.4: (top curve) DPPC control; (lower curves) DPPC plus PEI_bH_{50} at indicated concentrations.

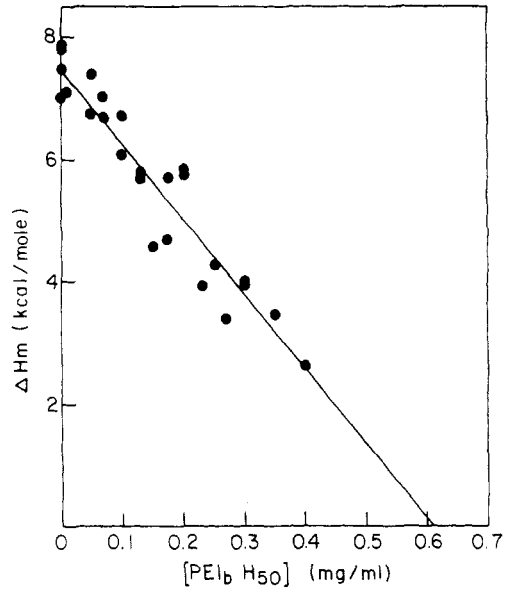

Figure 5. Dependence of DPPC main phase transition enthalpy (ΔH_m) on concentration of added PEI_bH_{50}.

toxin from *Naja mossambica mossambica*,[23] or proteolipid apoprotein of bovine myelin[24] causes a similar systematic reduction in lipid ΔH_m with increasing protein concentration, although the reduction is not always linear.[16,20,23] Explanations for these observations have taken two forms: (i) that the loss in ΔH_m reflects the withdrawal from the ordered lipid phase of a number of lipid molecules (typically 5–50) which are intimately associated with the hydrophobic protein (the so-called "boundary lipid"), or (ii) that crystallization of the lipid forces protein aggregation into protein-rich lipid "patches" which do not melt. In each of these explanations the integrity of the bilayer in the reconstituted system is assumed.

We must consider a third form of association for the PEI_bH_{50}/DPPC mixtures examined in this work. It cannot be assumed that the lipid bilayer remains intact; one can imagine that the hydrophobic PEI_bH_{50} might disrupt the

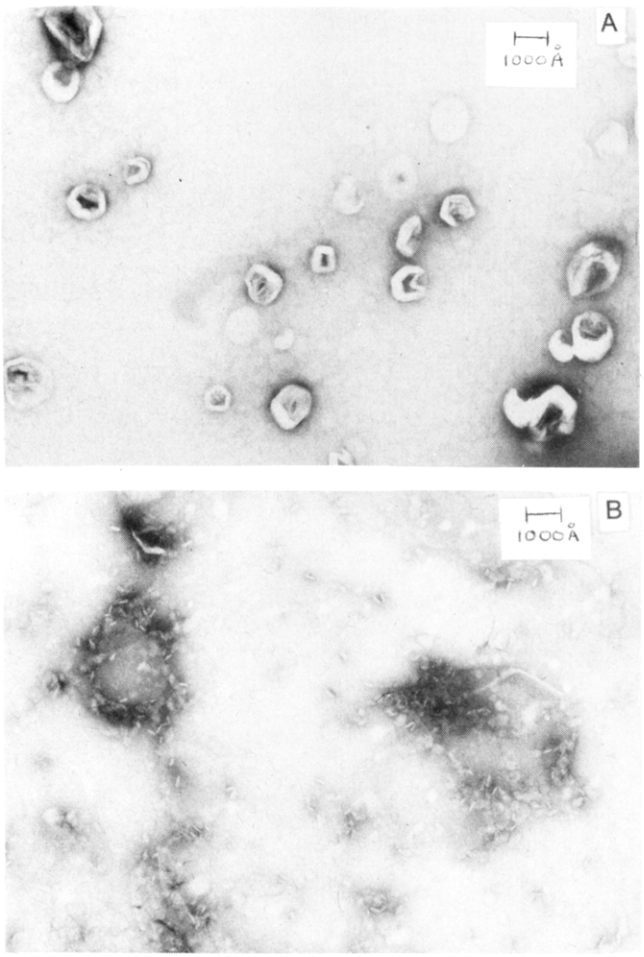

Figure 6. Negative-stain electron micrographs of DPPC dispersions prepared by mechanical shaking in Tris-HCl buffer, pH 7.4: (A) DPPC control, (B) DPPC plus PEI_bH_{50}.

bilayer entirely by nucleating the formation of a new polymer–lipid aggregate, perhaps a kind of mixed micelle. Mixing of the hydrophobic portions of the polymer and lipid molecules in such a micelle would prevent lipid crystallization, and an assumed stoichiometry of 0.60 mg of polymer per mg of DPPC in the micelle would produce the dependence of ΔH_m on polymer concentration shown in Figure 5.

The integrity of the lipid bilayer in systems such as these is an important question, which may have practical importance. We selected PEI for these experiments not only because it is related to the ionenes which we had studied previously and because it is readily prepared in linear and branched forms, but also because Klotz[10,25,26] and others[27,28] have shown that PEI_b serves as an excellent starting material for the construction of synthetic, enzyme-like catalysts of very high efficiency. One can imagine the incorporation of such catalysts into the bilayers of amphiphilic vesicles, with the result that chemical reactions accelerated by such catalysts would occur primarily within the bilayer. Appropriate functionalization of the vesicle surface[29] might then allow selective accumulation of reaction products within the vesicle interior, facilitating separation and recovery. We intend to pursue the development of such synthetic transmembrane catalysts.

The above speculation aside, the problem of the structure of the PEI_bH_{50}/DPPC aggregate remains. The molecular organization of the aggregate will require further study, but the gross morphology is apparent from negative-stain electron microscopy (Figure 6). Figure 6A is an electron micrograph of the control DPPC suspension and is consistent with the expected multilamellar vesicular structure of lipid suspensions prepared as in this work. Figure 6B is a similar micrograph of an aqueous mixture of DPPC and PEI_bH_{50} (1/1 by weight). The predominant structure in Figure 6B is that of a discoidal particle approximately 50 Å in thickness and 300 Å in diameter; large multilamellar vesicles similar to those in Figure 6A are absent.[30] It is interesting that similar discoidal particles, of similar size, have been observed in negative-stain electron micrographs of lipid suspensions reconstituted with hydrophobic proteins such as calcitonin[31] and apoprotein B of human plasma low-density lipoprotein.[32] Although it has been suggested by Walsh and Atkinson[32] that such particles are single-bilayer vesicles into which the protein is incorporated, we will defer speculation on the molecular organization of the PEI_bH_{50}/DPPC aggregates until ongoing structural studies are complete.

Conclusions

Unmodified poly(ethylenimines), branched or linear, interact strongly with the anionic phospholipid dipalmitoylphosphatidylglycerol, but not with the zwitterionic phospholipid dipalmitoylphosphatidylcholine, in aqueous suspension. Polymer chain branching modulates the poly(ethylenimine)–dipalmitoylphosphatidylglycerol interaction. Attachment of apolar side chains of 6–12 carbon atoms in length to branched poly(ethylenimine) produces derivatives which disrupt the bilayer organization of dipalmitoylphosphatidylcholine in a manner which is dependent on the length and concentration of the side chains. A branched poly(ethylenimine) bearing 50 mol % hexanoyl chains binds 1.7 mg of dipalmitoylphosphatidylcholine per mg of polymer, as determined from the decrease in lipid melting enthalpy with increasing polymer concentration. Electron microscopy of this system shows the formation of discoidal polymer–lipid aggregates 50 Å in thickness and 300 Å in diameter. These observations are parallel in many ways to those made previously in studies of the association of hydrophobic proteins with phospholipid bilayers.

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Registry No. DPPC, 63-89-8; dipalmitoylphosphatidylglycerol, 4537-77-3.

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Ene Reaction of (*S*)-(−)-4-(α-Methylbenzyl)-1,2,4-triazoline-3,5-dione with Propylene. X-ray Diffraction Analysis of a Single Crystal of the Brominated Adduct

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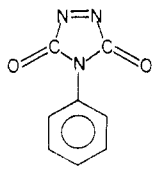
ABSTRACT: Optically pure (*S*)-(−)-4-(α-methylbenzyl)-1,2,4-triazoline-3,5-dione was synthesized and its ene reaction with propylene carried out in order to obtain a model compound for further study in the modification of polydienes. The product was brominated to demonstrate that the absolute configuration of the dione had been retained and to study the N–H hydrogen bonding. The compounds were fully characterized by using ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. A structural analysis of (*S*)-(−)-4-(α-methylbenzyl)-1-(2,3-dibromopropyl)urazole was performed via single-crystal X-ray diffraction. It crystallizes in space group $P2_12_12_1$ with four molecules in a unit cell of the following dimensions: a = 5.985 (2) Å, b = 10.758 (3) Å, c = 23.063 (3) Å, and V = 1484.8 (6) Å^3. The structure was solved by the heavy-atom method and refined by least-squares techniques to give a final R = 0.066. The dihedral angle between the phenyl ring and the five-membered ring is 65.8°, and oxygen is joined by the hydrogen bond to the N–H group [N(4)–H(4)—O(1)].

Introduction

There is recent interest in the 4-substituted-1,2,4-triazoline-3,5-diones, first synthesized by Thiele[1] in 1894. They are among the most powerful dienophiles and enophiles known. For example, 4-phenyl-1,2,4-triazoline-3,5-dione (**1**) has been shown to be 10^3 times more reactive



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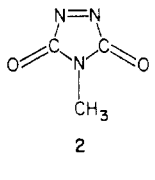

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with 2-chlorobutadiene than tetracyanoethylene (TCNE) and 2×10^3 times more reactive than maleic anhydride (MA).[2] Also, 4-methyl-1,2,4-triazoline-3,5-dione (**2**) has been found to undergo the Diels–ene reaction with cyclohexene at least 3×10^4 times faster than ethylazodicarboxylate.[3] We have investigated the reaction of **1** with a variety of organic substrates including enol ethers,[4] styrenes,[5] enol esters,[6] enols,[7] alkenes,[8] allyl silanes,[9] and poly(1,3-dienes).[10,11] Most of these reactions occur very rapidly at room temperature.

During the latter studies, it was found that poly(1,3-dienes) which had been modified to the extent of 1–5% by the ene reaction of the allylic function with **1** or **2** showed dramatic changes in their solubility character, thermal behavior, and tensile properties.[11] For example, these modified polymers exhibited dramatic decreases in

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