Chemistry and Physics of Lipids 196 (2016) 5–12

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Synthesis, characterization and thermotropic phase behavior of a homologous series of *N*-acyl-L-alaninols and interaction of *N*-myristoyl L-alaninol with dimyristoylphosphatidylcholine

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ARTICLE INFO

Article history:
Received 22 November 2015
Received in revised form 1 January 2016
Accepted 7 January 2016
Available online 29 January 2016

Keywords:
N-acyl-L-alaninol
Phase transition
Differential scanning calorimetry
Powder X-ray diffraction
Fluorescence spectroscopy
Transmission electron microscopy

ABSTRACT

N-acylethanolamines (NAEs) and their precursors, *N*-acylphosphatidylethanolamines are present in the cell membranes of a variety of species and exhibit interesting biological properties. *N*-acyl-L-alaninols (NAAOHs) are chiral homologues of NAEs and reduced forms of *N*-acyl-L-alanines (NAAs) and were reported to induce apoptosis in human lymphocytes. In the present study, we have synthesized and characterized a homologous series of *N*-acyl-L-alaninols ($n = 9 - 20$). In DSC studies in the dry as well as hydrated states NAAOHs with different chain lengths showed single sharp transitions similar to *N*-acyl-L-alanines. Transition enthalpies (ΔH_t) and entropies (ΔS_t) of NAAOHs are linearly dependent on the acyl chain length in both dry and hydrated states. Powder X-ray diffraction studies showed that the *d*-spacings of NAAOHs exhibit linear dependence on the chain length and the incremental increase in the *d* values suggest that they may be packed in a tilted bilayer pattern. Studies on the interaction of *N*-myristoyl L-alaninol (NMAOH) with DMPC revealed that the two amphiphiles mix well up to 45 mol% of NMAOH, whereas phase separation is observed at higher contents of the alaninol. Transmission electron microscopic studies show that the NMAOH:DMPC (45:55, mol/mol) mixture forms unilamellar vesicles of about 120–150 nm in diameter.

1. Introduction

Apoptosis is essential for the maintenance of normal homeostasis and is an important physiological response to many forms of stress e.g., ionizing radiation, UV light and chemotherapeutic agents (Haimovitze-Friedman et al., 1994). Ceramide has been termed as a tumor suppressing lipid, because of its apoptotic effect on cancer cells (Hannun, 1997). Currently, there is much interest in identifying other apoptosis inducing factors. Amino alcohols have been reported to suppress the proliferation of mouse B16 melanoma cells (Landau et al., 1993). Both D- and L- forms of *N*-acyl alaninols induce apoptosis in jurkat cells (human lymphocytes), which is similar to that induced by C2-ceramide and anti-Fas antibodies (Ogura and Handa, 2000). The D-isomer of *N*-myristoyl-phenylamino alcohol acts similar to C2-ceramide in inhibiting HL–60 leukemia cell growth, induces cell differentiation and acts as an inhibitor of alkaline ceramidases (Bielawska et al., 1992, 1996). *N*-palmitoyl serinols also induce apoptosis in neuroblastoma cells by increasing the C2 ceramide levels up to 50–80% (Bieberich et al., 2000). Apoptosis of human neuroblastoma, glioma, medulloblastoma, and adenocarcinoma cells is induced by *N*-acyl derivatives of serinol with palmitoyl (C16) and stearoyl (C18) acyl chains, diethanolamine with C16 acyl chains, and tris(hydroxy-methyl) methylamine with C18 acyl chain (Bieberich et al., 2002). The ceramide analogues *d*,*l*-threo-1-phenyl-2-acylamino-3-morpholino-1-propanol compounds act as potent inhibitors of *Plasmodium falciparum* growth in culture (Labaied et al., 2004).

The above observations indicate that *N*-acyl aminoalcohols are a new class of biochemically and pharmacologically important amphiphiles. In view of their amphiphilic nature, it is expected that

Abbreviations: NAAOHs, *N*-acyl-L-alaninols; NAEs, *N*-acylethanolamines; NAAs, *N*-acyl-L-alanines; DSC, differential scanning calorimetry; T_t, transition temperature; ΔH_t, transition enthalpy; ΔS_t, transition entropy; $\Delta H_{inc.}$, Incremental value contributed by each CH_2 group to transition enthalpy; $\Delta S_{inc.}$, incremental value contributed by each CH_2 unit to transition entropy; ΔH_o, end contribution to transition enthalpy; ΔS_o, end contribution to transition entropy; T_t^{∞}, transition temperature at infinite chainlength; PXRD, powder X-ray diffraction; NMAOH, *N*-myristoyl-L-alaninol; DMPC, dimyristoylphosphatidylcholine; TEM, transmission electron microscope.

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http://dx.doi.org/10.1016/j.chemphyslip.2016.01.001
0009-3084/

they would get incorporated into cell membranes and influence their phase properties. In addition, they may also influence the structural and dynamic properties of membrane proteins by directly interacting with them. Therefore, it is important to investigate their phase behavior and interaction with major membrane lipids, e.g., phosphatidylcholines, phosphatidylethanolamines and cholesterol as well as with membrane proteins. In the present study, we synthesized a homologous series of *N*-acyl-L-alaninols ($n = 9 - 20$) bearing saturated acyl chains and characterized their phase transitions in the dry and fully hydrated states and investigated the interaction of a representative *N*-acyl L-alaninol, namely NMAOH with a diacyl phosphatidylcholine with matching acyl chain length, DMPC. Powder X-ray diffraction studies suggest that in the solid state NAAOHs are most likely packed in a tilted bilayer structure. Studies on the interaction of NMAOH with DMPC revealed that the two lipids mix well up to 45 mol% NMAOH in the mixture. Transmission electron microscopic studies showed that near equimolar mixtures form vesicles, which might be useful in drug delivery applications.

2. Materials and methods

2.1. Materials

L-alaninol and fatty acids with different chain lengths (C9–C20) were purchased from Sigma–Aldrich (Germany). 1, 2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Laurdan was purchased from Molecular Probes (Invitrogen). Oxalyl chloride and other solvents were obtained from Merck (Mumbai, India). All other chemicals were purchased from local suppliers. Double distilled water was used in all experiments.

2.2. Synthesis of N-acyl-L-alaninols

N-acyl-L-alaninols bearing different acyl chains were synthesized by *N*-acylation of L-alaninol with the appropriate acid chloride using the procedure reported earlier for the synthesis of *N*-acylethanolamines (Ramakrishnan et al., 1997). Briefly, the acid chloride (1.0 mmol) was added dropwise to L-alaninol (4.0 mmol) in dichloromethane at 0 °C and the mixture was stirred for 3–4 h. The reaction mixture was then washed with water and brine solution, and the solvent was dried with anhydrous Na_2SO_4 and concentrated. The crude products were purified by recrystallization from hexane (for $n = 9 - 12$) or dichloromethane containing a trace of acetone (for $n = 13 - 20$) at −20 °C. The recrystallized products were characterized by IR, ^{1}H NMR, ^{13}C NMR and high resolution mass spectrometry (HRMS).

2.3. Differential scanning calorimetry

DSC experiments with dry samples were performed on a PerkinElmer Diamond differential scanning calorimeter. About 1–2 mg of each dry NAAOH was weighed accurately into an aluminum sample pan, covered with a lid and sealed by crimping. The reference pan was also prepared similarly but without any sample. For each sample three heating and two cooling scans were recorded at a scan rate of 2°/min. All compounds showed single sharp transitions in both heating and cooling scans; therefore the first heating scans were considered for further analysis. Transition enthalpies were determined by integrating the peak area under the transition curve. Transition entropies were calculated from the transition enthalpies assuming a first order transition, indicated by Eq. (1) (Marsh, 1990):

$$\Delta S_t = \frac{\Delta H_t}{T_t} \quad (1)$$

where T_t refers to the transition temperature and the ΔH_t values were taken at this temperature in order to calculate ΔS_t values.

For carrying out DSC measurements with hydrated samples, about 4–5 mg of each NAAOH was accurately weighed into a glass test tube and dissolved in ~300 μl of chloroform. A thin film of the lipid was obtained by gently blowing dry nitrogen gas over the solution. The lipid film was subjected to vacuum desiccation for about 6–8 h to remove the last traces of the solvent. The sample was then hydrated with 1 ml of double distilled water and vortexed. The hydrated lipid suspension was subjected to 5–6 cycles of freeze-thawing. Then 0.5 ml of lipid suspension was loaded in the sample cell of a VP–DSC equipment (MicroCal LLC, Northampton, MA, USA). For each sample three heating and two cooling thermograms were recorded at a scan rate of 60°/h. Although the transition temperatures were found to be reproducible in all the three heating scans, small decreases were observed in the transition enthalpies obtained in the second and third heating scans. Therefore, in all cases, the data from the first heating thermograms were used for further analysis. Transition temperatures were determined from the maxima of the transition peaks and transition enthalpies were estimated by integrating the area under the transition peaks using the Origin software provided by the DSC manufactures. Transition entropies were obtained from transition enthalpies using Eq. (1) assuming the transitions to be first order.

2.4. Powder X-ray diffraction studies

Powder X-ray diffraction measurements on NAAOHs were performed using a Bruker SMART D8 Advance powder X-ray diffractometer (Bruker-AXS, Karlsruhe, Germany) with Cu-Kα radiation at 40 kV and 30 mA. Finely powdered samples, obtained by grinding with mortar and pestle, were placed in the instrument sample holder. Diffraction data were collected using a LynxEye PSD data collector over a 2θ range of 1–50° at room temperature with a step size of 0.0198° and a measuring time of 1.5 s for each step. Peaks corresponding to $2\theta \leq 20°$ were used to calculate *d*-spacings employing Bragg's equation.

2.5. Interaction of N-myristoyl L-alaninol with DMPC

The interaction of NMAOH with DMPC was investigated by DSC. Samples were prepared by dissolving the two lipids in chloroform and mixing appropriate aliquots of the two solutions to give the desired mole ratio. The solvent was then evaporated by blowing dry nitrogen gas gently over the sample, and the final traces of solvent were removed by vacuum desiccation. Samples were hydrated with 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.4) containing 1 mM EDTA and 1 M NaCl. In order to obtain a homogeneous mixture the hydrated samples were subjected to 5 freeze–thaw cycles before DSC measurements. For DSC measurements, samples containing 10 mM of the total lipid mixture (NMAOH + DMPC) were loaded into the sample cell of the MicroCal VP–DSC equipment. For each sample three heating and two cooling thermograms were recorded between 5 °C and 85 °C at a scan rate of 60°/h. All samples were incubated for 30 min before the first heating scan. Although repeat scans yielded essentially identical heating thermograms for all samples, small decreases were seen in the transition enthalpies for the 2nd and 3rd heating scans. Therefore, the data for the first heating thermograms were taken for further analysis.