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Formation of high-axial-ratio-microstructures from natural and synthetic sphingolipids

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Abstract

Amphiphiles that form high-axial-ratio-microstructures (HARMs) are being considered as novel materials for controlled release of drugs and other biologically functional molecules. HARMs consisting of tubules, ribbons, solid rods and helices are formed from sphingolipids by addition of water to a solution of amphiphile in DMF. Single molecular species of galactocerebroside (GalCer) containing long unsaturated fatty acid chains or natural GalCer containing mixed-length, non-hydroxy fatty acids (NFA-GalCer) or α-hydroxy fatty acids (HFA-GalCer) form cylindrical structures. In contrast, single molecular species of GalCer containing long saturated fatty acids form ribbons and helices. GalCer HARMs are typically under 100 nm in diameter and have lengths of several microns. The importance of the amide of GalCer for HARM formation was evaluated using psychosine, which forms solid fibers, whereas sphingosine and an analog of GalCer in which the amide is reduced to a secondary amine form amorphous aggregates. Single molecular species of ceramide containing long unsaturated fatty acid chains form cylindrical structures, whereas those with long saturated fatty acids form ribbons and helices. Short chain saturated ceramide also forms cylindrical structures. GalCer analogs with *N*-acetyl-glycine in place of the galactose form fibers whereas those with *N*-acetyl-proline yield amorphous material. The *N*-acetyl-proline-containing amphiphile can de doped into pure GalCer or NFA-GalCer without perturbing tubule formation. © 1997 Elsevier Science Ireland Ltd.

Keywords: Cerebroside; Ceramide; Nanostructure; Tubule; Helix; Drug delivery

1. Introduction

Recently there have been reports demonstrating that two-chain amphiphiles can self-organize into

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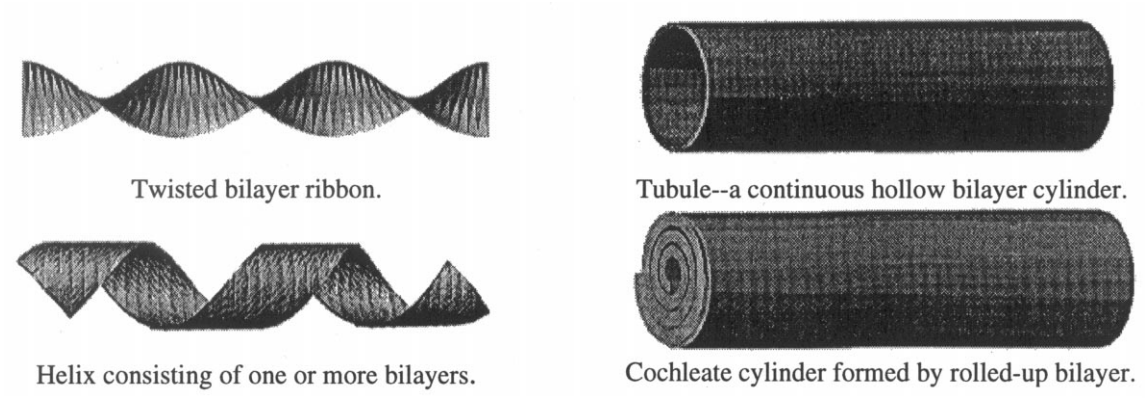

Fig. 1. Schematic images of tubules, cochleate cylinders, ribbons and helices.

stable, crystalline, nonliposomal microstructures when suspended in aqueous media (Yamada et al., 1984; Nakashima et al., 1984; Fuhrhop et al., 1988; Shimizu and Hato, 1993; Yager and Schoen, 1984; Georger et al., 1987). Such high-axial-ratio-microstructures (HARMs) include tubules, twisted ribbons, helices, and cochleate cylinders (Fig. 1). Theories on microstructure formation have been advanced for synthetic lipid systems such as phosphatidylcholine with butadiyne-containing hydrocarbon chains, and such theories have been applied to other HARM systems (Selinger et al., 1996; Nandi and Bagchi, 1996 and references therein). In short, such theories propose that HARM formation is the result of intrinsic bending of rectangular bilayer lipid sheets due to chiral packing of molecules in a membrane. Interest in natural and synthetic HARMs has stemmed primarily from their potential use as templates for mineralization and metallation, for their appearance in biological structures, for their implication in lipid storage diseases and as drug-delivery systems (Schnur et al., 1990; Behroozi et al., 1990; Chappell and Yager, 1992; Schnur et al., 1994; Mann et al., 1993; Rudolph et al., 1988; Archibald and Mann, 1994; Schnur et al., 1987). Such lipid molecular assemblies should have several advantages over current delivery systems: they require neither a macroscopic matrix nor a pump to achieve continuous drug release (by a zero-order kinetic dissolution process), they can limit drug exposure to the desired site of action and such assemblies may provide better shielding of the drug from premature metabolic degradation.

Sphingolipids have been reported to form HARMs (Kulkarni et al., 1995; Archibald and Mann, 1993; Archibald and Yager, 1992; Curatolo and Neuringer, 1986). Cerebrosides are the simplest mammalian glycosphingolipids. These lipids consist of a galactose or glucose headgroup attached to a nonpolar ceramide. Due to order-disorder transition temperatures (T_m) well above body temperature, cerebrosides are thought to impart order to membranes (Curatolo and Neuringer, 1986). In lipid storage diseases such as Gaucher's and Krabbe's disease, cerebroside accumulation disrupts normal membrane function and forms intracellular lipid deposits (Naito et al., 1988). Such deposits give rise to microstructures within the afflicted cells (Yunis and Lee, 1970). Furthermore, galactocerebroside (GalCer) is a major component of myelin and the intestinal brush border and thus contributes to these membrane's physical properties. Ceramide (Cer), the product of hydrolytic removal of the sugar from cerebroside, is thought to serve in cell signal transduction (Bielawska et al., 1996). Further hydrolysis yields, in addition to fatty acids, sphingosine, which also participates in cell signaling (Bielawska et al., 1996).

Earlier investigations into the ability of sphingolipids to form microstructures focused on the development of new formation techniques and, to a limited extent, pure GalCer species. In this paper, we demonstrate that it is possible to form

HARMs from various pure GalCer and Cer species using a new formation technique. Furthermore, synthesized GalCer analogs were converted to microstructures either as single molecular species or as a component of binary mixtures. These studies are a necessary prelude to understanding the basic structural requirements for sphingolipid molecular assemblies.

2. Experimental procedures

2.1. Materials

All materials were of reagent grade purity and used as received. D-erythro-sphingosine was purchased from Avanti Polar Lipids. All other non-synthesized lipids were purchased from Sigma (St. Louis, MO). ^{1}H NMR spectra were obtained in $CDCl_3$ using a Bruker 300 MHz or 500 MHz NMR spectrometer with tetramethylsilane as an internal standard. Silica gel (EM Science Silica Gel 60, 230-400 Mesh) was used for all flash chromatography. Thin layer chromatography was performed using Silica Gel 60 F254 (EM Science).

2.1.1. Microscopy

Phase contrast optical micrographs were taken using a Zeiss ICM 405 microscope (Carl Zeiss, Thornwood, NY) with 40 × (NA 0.75) phase contrast lenses. Transmission electron micrographs (TEM) were obtained using a Philips EM 410 electron microscope operating at an acceleration potential of 80 kV. Samples were applied to Formvar-coated 150 mesh copper TEM sample grids with and without negative stain (2% aqueous ammonium molybdate pH 5.0).

2.1.2. Calorimetery

Differential scanning calorimetery was performed using a Seiko DSC-100 high sensitivity calorimeter. Known concentrations of aqueous lipid suspensions (50 μl) were heated from 1–95°C at 1°C/min in 70 μl silver calorimetery pans. In order to insure proper hydration of the lipids, each sample was pre-heated under the same conditions before the actual run. After calorimetery, the pan's contents were analyzed for decomposition by thin layer chromatography.

2.2. Chemical synthesis

The chemical synthesis of amphiphiles not commercially available is shown in Fig. 2.

2.2.1. N-hydroxysuccinimide ester of nervonic acid

The *N*-hydroxysuccinimide ester of nervonic acid was prepared according to Lapidot et al. (1967): Nervonic acid (0.558 g, 1.52 mmol) and *N*-hydroxysuccinimide (0.175 g, 1.52 mmol) in 60 ml anhydrous EtOAc were stirred overnight with dicyclohexylcarbodiimide (0.314 g, 1.52 mmol). The white precipitate was removed, and the supernatant evaporated in vacuo. The residue was recrystallized from EtOH to provide the *N*-hydroxysuccinimide ester of nervonic acid as fine white needles (0.539 g, 76%): m.p. 58–60°C; R_f ($CHCl_3$) 0.24; ^{1}H NMR (500 MHz) 5.35 (t, 2H, C-15, C-16, J = 5.0 Hz), 2.81 (d, 4H, succinimide, J = 4.5 Hz), 2.60 (t, 2H, C-2, J = 7.6 Hz), 2.01 (m, 4H, C-14, C-17), 1.74 (t, 2H, C-3, J = 5.5 Hz), 0.88 (t, 3H, C-24, J = 7.0 Hz).

2.2.2. N-nervonoyl ceramide (24:1-cer)

N-nervonoyl ceramide was prepared according to Ong and Brady (1972): The *N*-hydroxysuccinimide ester of nervonic acid (0.092 g, 198.4 μmol)

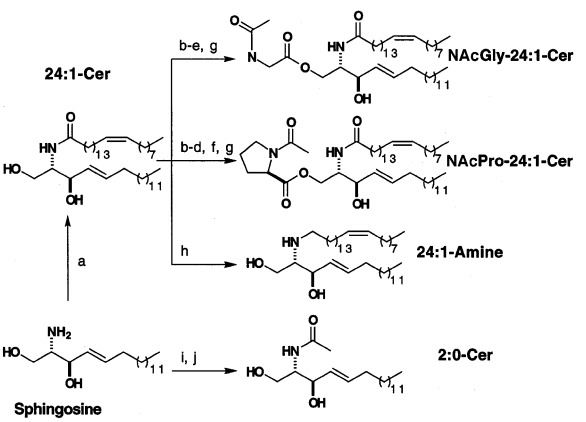

Fig. 2. Chemical synthesis of amphiphiles not commercially purchased.

and sphingosine (0.062 g, 207.0 μmol) were dissolved in 10 ml anhydrous THF and stirred overnight under Ar. Flash chromatography (1:0:0-90:10:1 $CHCl_3$:MeOH:NH_4OH) provided *N*-nervonoyl ceramide as a white solid (0.118 g, 91%): R_f (9:1 $CHCl_3$:MeOH) 0.47; ^1H NMR (500 MHz) 6.22 (d, 1H, NH, J = 7.1 Hz), 5.73 (m, 1H, C-5), 5.53 (dd, 1H, C-4, J = 6.3, 15.4 Hz), 5.34 (t, 2H, C-15′, C-16′, J = 4.6 Hz), 4.30 (t, 1H, C-3, J = 3.8 Hz), 3.96 (dd, 1H, C-1, J = 3.1, 11.0 Hz), 3.91 (m, 1H, C-2), 3.71 (dd, 1H, C-1, J = 3.1, 11.0 Hz), 2.22 (t, 2H, C-2′, J = 7.4 Hz), 2.00 (m, 6H, C-6, C-14′, C-17′), 1.60 (t, 2H, C-3′, J = 7.8 Hz), 0.88 (t, 6H, C-18, C-24′, J = 6.3 Hz).

2.2.3. *N*-nervonoyl-1-0-triphenylmethyl ceramide

N-nervonoyl ceramide (0.018 g, 27.8 mmol), triphenylmethyl chloride (0.015 g, 55.5 mmol) and *N*,*N*-dimethyl-4-aminopyridine (0.007 g, 55.5 mmol) in 20 ml anhydrous toluene were refluxed for 16 h under Ar. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (9:1-1:1 hexane:EtOAc) to provide *N*-nervonoyl-1-0-triphenylmethyl ceramide as a white solid (0.018 g, 72%): R_f (3:1 hexane:EtOAc) 0.21; ^1H NMR (300 MHz) 7.42-7.22 (m, 15H), 6.06 (d, 1H, NH, J = 7.9 Hz), 5.63 (m, 1H, C-5), 5.35 (t, 2H, C-15′, C-16′, J = 5.2 Hz), 5.25 (dd, 1H, C-4, J = 6.2, 15.5 Hz), 4.18 (m, 1H, C-2), 3.69 (dd, 1H, C-3, J = 3.9, 7.8 Hz), 3.32 (m, 2H, C-1), 2.20 (t, 2H, C-2′, J = 8.1 Hz), 2.00 (m, 4H, C-14′, C-17′), 1.91 (m, 2H, C-6), 1.64 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′, J = 6.5 Hz).

2.2.4. *N*-nervonoyl-1-0-triphenylmethyl-3-*O*-[*t*-butyldiphenylsilyl] ceramide

The title compound was prepared according to Numata et al. (1988): *N*-nervonoyl-1-*O*-triphenylmethyl ceramide (0.108 g, 0.12 mmol), imidazole (0.066 g, 0.97 mmol), and *t*-butylchlorodiphenylsilane (0.79 ml, 3.03 mmol) were stirred 19.5 h in 25 ml anhydrous DMF under Ar. Twenty five ml of H_2O was added and the mixture extracted with Et_2O (3 × 15 ml). The ether extracts were washed with 10 ml H_2O and 10 ml saturated NaCl (aq). Flash chromatography (15:1-2:1 hexane:EtOAc and 1 ml triethylamine/100 ml of solvent) provided the title compound as a white solid (0.090 g, 66%): R_f (3:1 hexane:EtOAc) 0.66; ^1H NMR (300 MHz) 7.70–7.23 (m, 25H), 5.36–5.25 (m, 5H, NH, C-4, C-5, C-15′, C-16′), 4.39 (t, 1H, C-3, J = 5.4 Hz), 4.18 (m, 1H, C-2), 3.94 (dd, 1H, C-1, J = 5.1, 10.4 Hz), 3.70 (dd, 1H, C-1, J = 5.1, 10.4 Hz), 2.00 (m, 4H, C-14′, C-17′), 1.86 (m, 2H, C-2′), 1.72 (m, 2H, C-6), 1.44 (m, 2H, C-3′), 1.04 (s, 9H, *t*-Bu), 0.88 (t, 6H, C-18, C-24′, J = 7.3 Hz).

2.2.5. *N*-nervonoyl-3-*O*-[*t*-butyldiphenylsilyl] ceramide

Compound 2.2.4. was detritylated according to Koike et al. (1986): *N*-nervonoyl-1-0-triphenylmethyl-3-*O*-[*t*-butyldiphenylsilyl] ceramide (0.093 g, 82.4 μmol) was stirred for 4 h with *p*-toluenesulfonic acid monohydrate (0.010 g, 49.4 μmol) in 20 ml 1:1 MeOH:CH_2Cl_2. Et_2O (40 ml) was added, and the solution was washed with 10 ml 5% $NaHCO_3$ (aq) and 10 ml H_2O. Flash chromatography (6:1-0:1 hexane:EtOAc) provided product as a white solid (0.034 g, 47%): R_f (3:1 hexane:EtOAc) 0.15; ^1H NMR (500 MHz) 7.67–7.30 (m, 10H), 5.93 (d, 1H, NH, J = 7.1 Hz), 5.42–5.33 (m, 4H, C-4, C-5, C-15′, C-16′), 4.34 (t, 1H, C-3, J = 4.5 Hz), 3.97-3.82 (m, 2H, C-1, C-2), 3.60 (m, 1H, C-1), 3.14 (m, 1H, OH), 1.98 (m, 6H, C-2′, C-14′, C-17′), 1.86 (m, 2H, C-6), 1.55 (m, 2H, C-3′), 1.07 (s, 9H, *t*-Bu), 0.88 (t, 6H, C-18, C-24′, J = 7.0 Hz).

2.2.6. *N*-nervonoyl-1-*O*-(*N*-acetyl-glycine)-3-*O*-[*t*-butyldiphenylsilyl] ceramide

Amino acylation was adapted from the procedure of Neises and Steglich (1985): *N*-nervonoyl-3-*O*-[*t*-butyldiphenylsilyl] ceramide (0.021 g, 23.7 μmol), *N*-acetyl-glycine (0.006 g, 47.4 μmol), and *N*,*N*-dimethyl-4-aminopyridine (0.06 g, 47.4 mmol) in 21 ml 2:5 CH_3CN:CH_2Cl_2 (anhydrous) were stirred for 2 h under Ar. Dicyclohexylcarbodiimide (0.010 g, 47.4 mmol) was added and the reaction stirred for 24 h under Ar. The solvents were removed in vacuo. Flash chromatography (5:1-0:1 hexane:EtOAc) of the residue provided product as a white solid (0.016 g, 70%):

R_f (1:1 hexane:EtOAc) 0.23; ^{1}H NMR (300 MHz) 7.67–7.57 (dd, 4H), 7.46–7.33 (m, 6H), 6.09 (bs, 1H, NH), 5.51–5.29 (m, 4H, C-4, C-5, C-15′, C-16′), 4.40 (dd, 1H, C-3, J = 2.9, 10.8 Hz), 4.26 (bs, 2H, C-1), 4.12 (m, 1H, C-2), 3.93 (t, 2H, glycine, J = 11.3 Hz), 2.00 (s, 3H, NAc), 1.05 (s, 9H, *t*-Bu), 0.88 (t, 6H, C-18, C-24′, J = 6.4 Hz).

2.2.7. *N*-nervonoyl-1-*O*-(*N*-acetyl-l-proline)-3-*O*-[*t*-butyldiphenylsilyl] ceramide

N-nervonoyl-3-*O*-[*t*-butyldiphenylsilyl] ceramide (0.034 g, 38.4 μmol), *N*-acetyl-L-proline (0.010 g, 63.6 μmol), and *N*,*N*-dimethyl-4-aminopyridine (0.011 g, 90.0 μmol) in 15 ml 1:2 CH_3CN:CH_2Cl_2 (anhydrous) were stirred for 30 min under Ar. Dicyclohexylcarbodiimide (0.012 g, 57.5 μmol) was added and the reaction stirred for 24 h under Ar. The white precipitate was removed by vacuum filtration and the solvents evaporated in vacuo. Flash chromatography (6:1-0:1 hexane:EtOAc) of the residue provided product as a white solid (0.029 g, 74%): R_f (1:1 hexane:EtOAc) 0.29; ^{1}H NMR (300 MHz) 7.68–7.59 (dd, 4H), 7.43–7.26 (m, 6H), 6.14 (d, 1H, NH, J = 8.8 Hz), 5.41–5.29 (m, 3H, C-4, C-15′, C-16′), 5.14 (dt, 1H, C-5, J = 4.0, 8.8 Hz), 4.69 (d, 1H, α, J = 7.7 Hz), 4.39 (dd, 1H, C-3, J = 3.6, 8.1 Hz), 4.27 (d, 2H, C-1, J = 12.4 Hz), 4.02 (t, 1H, C-2, J = 7.3 Hz), 3.44 (t, 2H, δ, J = 6.4 Hz), 2.16 (m, 2H, β), 2.02-1.91 (m, 13H, C-6, C-2′, C-14′, C-17′, γ, NAc), 1.49 (m, 2H, C-3′), 1.03 (s, 9H, *t*-Bu), 0.88 (t, 6H, C-18, C-24′, J = 6.6 Hz).

2.2.8. *N*-nervonoyl-1-*O*-(*N*-acetyl-glycine) ceramide (NAcGly-24:1-Cer)

Desilylation was performed according to Hanessian and Lavallee (1975): *N*-nervonoyl-1-*O*-(*N*-acetyl-glycine)-3-*O*-[*t*-butyldiphenylsilyl] ceramide (0.009 g, 9.1 μmol) in 10 ml anhydrous THF and 0.01 ml 1.0 M *n*-butylammonium fluoride in THF were stirred for 1 h under Ar. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (2:1-0:1 hexane:EtOAc) to provide compound product as a white solid (0.002 g, 29%): R_f (EtOAc) 0.25; ^{1}H NMR (500 MHz) 6.11 (bs, 1H, NH), 6.01 (bs, 1H, NH), 5.76 (dt, 1H, C-5, J = 6.7, 15.5), 5.48 (dd, 1H, C-4, J = 6.2, 15.5 Hz), 5.33 (t, 2H, C-15′, C-16′, J = 5.0 Hz), 4.33 (d, 2H, gly), 4.15 (m, 2H, C-2, C-3), 4.00 (m, 2H, C-1), 2.17 (t, 2H, C-2′, J = 4.4 Hz), 2.03 (s, 3H, NAc), 0.86 (t, 6H, C-18, C-24′, J = 6.6 Hz).

2.2.9. *N*-nervonoyl-1-*O*-(*N*-acetyl-L-proline) ceramide (NAcPro-24:1-Cer)

Desilylation was performed according to Hanessian and Lavallee (1975): *N*-nervonoyl-1-*O*-(*N*-acetyl-L-proline)-3-*O*-[*t*-butyldiphenylsilyl] ceramide (0.021 g, 20.5 μmol) in 12 ml anhydrous THF and 0.01 ml 1.0 M *n*-butylammonium fluoride (in THF) were stirred for 2 h under Ar. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (3:1-0:1 hexane:EtOAc) to provide product as a white solid (0.011 g, 69%): R_f (EtOAc) 0.31; ^{1}H NMR (500 MHz) 6.66 (d, 1H, NH, J = 7.7 Hz), 5.70 (dt, 1H, C-5, J = 6.7, 15.5), 5.47 (dd, 1H, C-4, J = 6.2, 15.5 Hz), 5.32 (t, 2H, C-15′, C-16′, J = 4.6 Hz), 4.47-4.26 (m, 4H, α, C-2, C-3), 4.06 (bs, 2H, C-1), 3.64-3.50 (dm, 2H, δ), 3.30 (bs, 1H, OH), 2.18 (m, 2H, β), 2.07 (s, 3H, NAc), 1.99 (m, 10H, C-6, C-2′, C-14′, C-17′, γ), 1.59 (m, 2H, C-3′), 0.86 (t, 6H, C-18, C-24′, J = 7.0 Hz).

2.2.10. *N*-tetracos-15(Z)-enyl-sphingosine (24:1-Amine)

Nervonoyl ceramide (0.015 g, 23.1 μmol) was dissolved in 12 ml anhydrous Et_2O. Lithium aluminum hydride (0.021 g, 485.8 μmol) was added, and the mixture was refluxed for 1.5 h and then stirred at room temperature for 48 h. Excess hydride reagent was quenched by addition of 3 ml EtOAc followed by 1 drop of saturated Na_2SO_4 (aq). The resultant white precipitate was filtered and the solution purified by flash chromatography (1:0-20:1 $CHCl_3$:MeOH) to provide 24:1-Amine as a white solid (0.051 g, 33%): R_f (9:1 $CHCl_3$:MeOH) 0.19; ^{1}H NMR (500 MHz) 5.87 (dt, 1H, C-5, J = 6.7, 15.5), 5.45 (dd, 1H, C-4, J = 5.7, 15.5 Hz), 5.35 (t, 2H, C-15′, C-16′, J = 4.9 Hz), 4.69 (bs, C-3), 4.05 (dd, 1H, C-1, J = 4.6, 12.9 Hz), 3.90 (dd, 1H, C-1, J = 4.6, 12.9 Hz), 3.46 (m, 1H, NH), 2.95 (m, 1H, C-2), 2.08-1.99 (m, 6H, C-6, C-14′, C-17′), 0.88 (t, 6H, C-18, C-24′, J = 6.7 Hz).

2.2.11. N-acetyl ceramide (2:0-Cer)

Sphingosine (0.008 g, 26.7 μmol) in 10 ml 1:1 CH_2Cl_2:pyridine and 1 ml acetic anhydride were stirred for 4.5 h and then evaporated in vacuo. The residue was dissolved in 10 ml MeOH, and K_2CO_3 (0.025 g, 180.8 μmol) was added. After stirring overnight, the solvent was removed in vacuo and the residue purified by flash chromatography (1:0-20:1 $CHCl_3$:MeOH) to provide product as a white solid (0.004 g, 44%): R_f (9:1 $CHCl_3$:MeOH) 0.35; ^{1}H NMR (300 MHz) 6.32 (d, 1H, NH), 5.78 (dt, 1H, C-5, J = 6.7, 15.5), 5.50 (dd, 1H, C-4, J = 3.6, 15.5 Hz), 4.35 (bt, 1H, C-3, J = 5.8 Hz), 3.97 (dd, 1H, C-1, J = 3.5, 11.2 Hz), 3.93 (m, 1H, C-2), 3.71 (dd, 1H, C-1, J = 3.5, 11.2 Hz), 2.30 (t, 3H, C-6, J = 9.0 Hz), 2.05 (m, 7H, NAc, C-14′, C-17′), 0.89 (t, 3H, C-18 J = 3.4 Hz).

2.3. Microstructure formation studies

Several methods were employed to determine the tendency of the lipids to form HARMs.

2.3.1. DMF/H_2O

Amphiphile (0.1 mg) was dissolved in anhydrous DMF so that the concentration was 1.0 mM. Water was added in $\approx$10 μl increments until the solution became cloudy. The resulting suspensions were incubated at 20°C for 2–24 h. For larger amounts of amphiphile, water was added with vortex mixing ($\approx$3 s) between additions.

2.3.1.1. DMF/saline. To NFA-GalCer dissolved in DMF (0.8 mg/ml) was added a saturated saline solution of LiBr, LiCl, NaCl or $MgCl_2$ (50% by volume) while hand swirling. The resultant white gel was incubated at 20°C for 16 h.

2.3.2. Pyridine evaporation

Amphiphile (0.1 mg) was dissolved in pyridine so that the concentration was 1.0 mM. Water was added in $\approx$10 μl increments until the solution became cloudy. The samples were incubated at 20°C so that solvent evaporated and a precipitate formed (typically 24–48 h).

2.3.3. Freeze-thaw

This procedure follows the method of Kulkarni et al. (1995): Amphiphile (0.1 mg) was suspended in 1 ml of aqueous buffer (10 mM KH_2PO_4, 100 mM NaCl, 1.5 mM NaN_3, pH = 6.6). The suspension was thrice incubated for 3 min at 90°C, vortexed for 20 s at room temperature and then sonicated (Laboratory Supplies and Company, Hicksville, NY, output 80 KC) for 20 s at room temperature. Next, the suspension was placed in *i*-PrOH/dry ice for 2 min, thawed rapidly ($\approx$20 s) and then vortexed 20 s. The freeze-thaw procedure was repeated three times except that after the last freeze the sample was allowed to warm to room temperature over 1.5 h.

2.3.4. Thermal cycling

This procedure follows the method of Archibald and Yager (1992): amphiphile was placed in ethylene glycol:water (either 19:1) to a final concentration of 1 mg/ml. The suspension was thrice incubated for 10 min at 99°C and sonicated at 50°C (12 × 30 s pulses with 30 s pauses). After the final sonication, the suspension was allowed to cool from 99°C to room temperature over 2.5 h.

2.4. Mixed lipid studies

2.4.1. NAcPro-24:1-Cer: 24:1-GalCer

Particles from the mixtures of NAcPro-24:1-Cer and 24:1-GalCer (1:1, 1:3, 1:5, 1:7 mol:mol) were prepared as in Section 2.3.1 and incubated for 24 h at 20°C. In order to determine the ratio of the lipids incorporated in the HARMs, samples were pelleted (2500 g for 30 min) and a known percentage of the supernatants removed. Supernatants and pellets were dried in vacuo and the residues weighed (mass corrections were made for the portion of supernatant that remained with the pellet).

2.4.1.1. Qualitative assessment of incorporation. Supernatant and pellet material (from Section 2.4.1) was heavily spotted on silica TLC plates (4 cm length) and developed in EtOAc. The plates were stained with phosphomolybdic acid reagent (Aldrich, Milwaukee, WI) and then heated (NAcPro-24:1-Cer R_f = 0.31 and 24:1-GalCer R_f = 0.0).

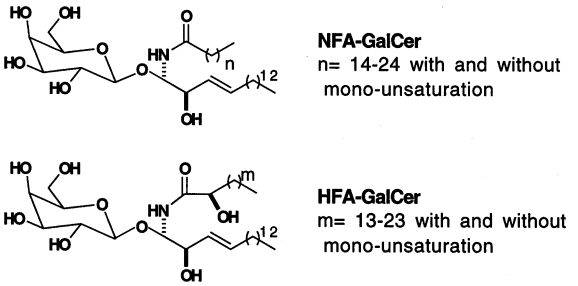

Fig. 3. The subfractions of Galactocerebroside.

2.4.1.2. A quantitative assessment of incorporation. The dried and weighed 1:3 doped supernatant and pellet (Section 2.4.1) were dissolved in CD_3OD and the amphiphile ratio was determined by comparison of the integrands of the NAcPro-24:1-Cer αH (δ 4.5) and vinyl NMR signals.

2.4.2. NAcPro-24:1-Cer : 24:1-Cer

The procedure was identical to the NAcPro-24:1-Cer: nervonoyl-GalCer mixed lipid study (Section 2.4.1). Compositional NMR analysis of the amphiphile ratio was effected by comparison of the integrands of NAcPro-24:1-Cer αH (δ 4.5) and C-4 vinyl (δ 5.4) in CD_3OD.

2.4.3. NAcPro-24:1-Cer : NFA-GalCer

Performed as per NAcPro-24:1-Cer: nervonoyl-GalCer mixing studies (Section 2.4.1).

3. Results and discussion

3.1. HFA-GalCer and NFA-GalCer

GalCer can be divided into two major subfractions, that containing non-hydroxy fatty acids (NFA-GalCer) and that containing more polar α-hydroxy fatty acids (HFA-GalCer) (Fig. 3). The ability of GalCer to self-organize has been previously investigated using pyridine evaporation, thermal and freeze-thaw cycling techniques to form microstructures. Archibald and Yager found that submission of HFA-GalCer to pyridine evaporation or thermal cycling resulted in the formation of cochleate cylinders of dimensions $5–30 \times 100–300$ nm, whereas NFA-GalCer formed tubules and helical ribbons using pyridine evaporation or thermal cycling (Archibald and Yager, 1992). Brown and coworkers, using a freeze-thaw method, found that NFA-GalCer formed tubules and multilamellar liposomes (Kulkarni et al., 1995). All of these observations were confirmed in the present study.

In the present study, we developed a DMF/H_2O precipitation method for the exploration of HARM formation. Application of this technique to NFA-GalCer yields cylindrical structures (Fig. 4a). Submission of HFA-GalCer to aggregate formation using DMF/H_2O provides cylindrical structures with some helical ribbon content (Fig. 4b). This is in stark contrast to the cochleate cylinders produced by thermal cycling of the same amphiphile (Fig. 4c). Depending on the formation methodology the HFA-GalCer HARMs have different dimensions (Table 1). This implies that the formation methodology/solvent can affect HARM morphology.

The differences in tubule diameter and length may be influenced by solvent, electrolytes, lipid concentration and variations in composition of NFA-GalCer and HFA-GalCer. The freeze-thaw method uses 100 mM NaCl whereas the DMF/H_2O method is carried out in the absence of salt. An early tubule formation theory argues that a tilted phase of chiral molecules must be electrostatically polarized which, in turn, can cause a membrane strip to collapse upon itself and form a cylinder (de Gennes, 1987). In this theory salts should increase the tubule radius because electrostatic interactions would be shielded. Along this line, Archibald noted that the dimensions of HFA-GalCer cochleate cylinders formed by thermal cycling were larger when formed in the presence of salt (Archibald and Yager, 1992). Furthermore, in the better studied HARMs composed of phosphatidylcholine with butadiyne containing hydrocarbon chains, the tubule outer diameter increased as the salt concentration was increased in the formation medium, but this is possibly the result of more bilayers in the tubule

Table 1
Effect of formation methodology/solvent on HARM morphology

Amphiphile	T_m (°C)[a]	Method[b]	Structure	Size (nm)[c]	Reference
NFA-GalCer	69.5	Pyridine	Tube/ribbon	$85 \times \geq 10\,000$	Archibald and Yager (1992)
		Thermal	Tube/ribbon	$85 \times \geq 10\,000$	Archibald and Yager (1992)
		Freeze	Tube/liposome	$40–100 \times 500–5000$	Kulkarni et al. (1995)
		Freeze	Cochleate	$100–150 \times \geq 1000$	Kulkarni and Brown (1996)
	70.5	DMF(35)	Tube	25–50	
HFA-GalCer		Pyridine	Cochleate	$5–30 \times 10\,000–30\,000$	Archibald and Yager (1992)
		Thermal	Cochleate	$5–30 \times 10\,000–30\,000$	Archibald and Yager (1992)
	66.1	DMF(35)	Tube/ribbon	$40–100 \times 2000–7000$	
24:1-GalCer	59.3	Freeze	Tube	$25–30 \times 250–400$	Kulkarni et al. (1995)
		DMF(12)	Tube	20×600	
22:1-GalCer		Freeze	Tube	25–35	Kulkarni and Brown (1996)
20:1-GalCer		Freeze	Ribbon	40–50	Kulkarni and Brown (1996)
18:1-GalCer		Freeze	Liposome/sheet/ multilamellar tubes		Kulkarni and Brown (1996)
	45.4	DMF(35)	Tube	30–60	
24:0-GalCer	84.1	Freeze	Ribbon	$100–250 \times 2000–10\,000$	Kulkarni et al. (1995)
18:0-GalCer	81.1	DMF(35)	Ribbon	$8–16 \times 1250$	
16:0-GalCer		H_2O	Tube	100	Curatolo and Neuringer (1986)
		DMF(35)	Ribbon	24	
Psychosine		Thermal	Amorphous		Archibald and Yager (1992)
	40.1	DMF(105)	Fiber	$50 \times 13\,000$	
Sphingosine		MeOH(12)	Cochleate	50	Archibald and Mann (1993)
	42.3	DMF(150)	Amorphous		
Ceramide		Thermal	Amorphous		Archibald and Yager (1992)
24:1-Cer		Thermal	Amorphous		
		Pyridine	Amorphous		
	71.8	DMF(3)	Tube	$55–140 \times 40\,000$	
18:1-Cer	48.3	DMF(70)	Tube	19–24	
18:0-Cer		DMF(35)	Ribbon	8–11	
16:0-Cer		DMF(35)	Ribbon	11–25	
6:0-Cer	51.2	DMF(70)	Tube	50–75	
2:0-Cer	49.3	DMF(30)	Amorphous		
24:1-Amine	36.5	DMF(20)	Amorphous		
NAcPro-24:1-Cer	42.0	Pyridine	Amorphous		
		Thermal	Amorphous		
		Freeze	Amorphous		
		DMF(16)	Amorphous		
NAcGly-24:1-Cer		DMF(30)	Tube	110–200	

[a] T_m measurements refer to the major transition.
[b] Pyridine refers to pyridine evaporation method. Thermal and freeze refer to thermal and freeze-thaw cycling methodologies, respectively. DMF refers to DMF/H_2O precipitation. MeOH refers to MeOH/H_2O precipitation. H_2O refers to suspension of lyophilized powder in water. The number in parenthesis refers to the volume percentage of water added.
[c] If no second measurement is provided then the length was indeterminate.

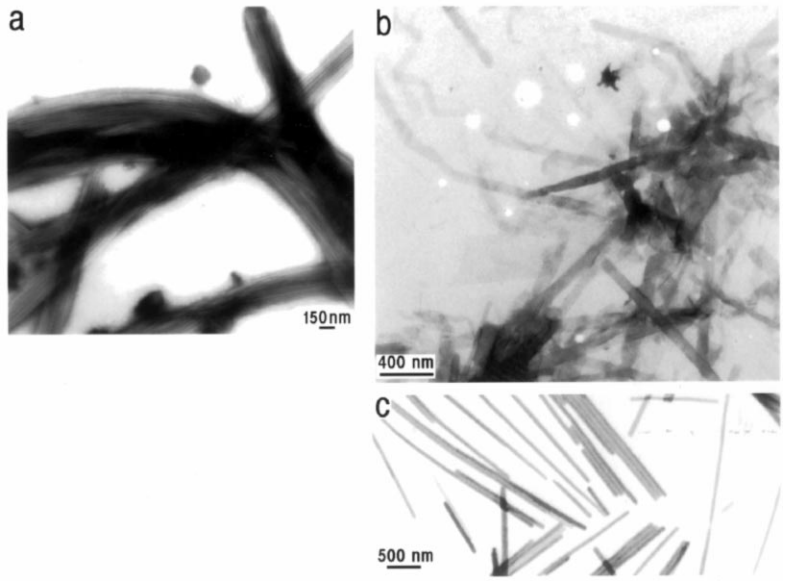

Fig. 4. Negatively stained TEM images of: (a) NFA-GalCer HARMs formed by DMF/H_2O precipitation; (b) HFA-GalCer HARMs formed by DMF/H_2O precipitation shows some helical ribbon content in addition to cylindrical structures; (c) HFA-GalCer formed by thermal cycling.

(Chappell and Yager, 1991). In the present study, it was found that precipitation of NFA-GalCer from DMF by addition of saturated saline solutions (LiBr, LiCl, NaCl, $MgCl_2$) rather than pure water still forms cylindrical structures; however, the tubules are irregularly shaped but, on average, the dimensions remain unchanged (not shown).

The lipid concentration should not perturb tubule formation as long as the lipid concentration is above the critical micelle concentration (CMC). In all cases, cerebroside concentration was above 12.3 μM. Although not measured, cerebroside CMC values should be vanishingly low (less than nanomolar) due to high, long-chain fatty acid content, thus transfer of lipid to crystalline HARMs from other fluid lipid structures, at room temperature, should be prohibitively slow. On the other hand, lipid concentration may affect tubule wall thickness (Spector et al., 1996). As shown in studies involving solvent precipitation of phosphatidylcholine containing butadiyne hydrocarbon chains, more concentrated lipid solutions formed wider tubules due to multiple bilayer wrappings. Contrary to this, 24:1-GalCer tubules prepared by DMF/H_2O precipitation are narrower than Brown's freeze-thaw tubules (described later) despite being formed from a more concentrated solution (Kulkarni et al., 1995). Lastly, natural variations in HFA- and NFA-GalCer fatty acid composition and unsaturation may affect HARM formation and dimensions.

3.2. Pure galactocerebrosides

To better understand the structural requirements of cerebrosides to form HARMs, single molecular specifies of GalCer-based amphiphiles were studied. Using DMF/H_2O precipitation, pure 24:1-GalCer forms cylindrical nanostructures of dimensions 20×600 nm as evidenced by negative stained TEM (Fig. 5a, Table 1). Using freeze-thaw cycling, Brown found that 24:1-GalCer formed tubules, but these are shorter and wider than those formed by the DMF/H_2O method (Table 1) (Kulkarni et al., 1995).

Replacement of the mono-unsaturated, 24-carbon fatty acid chain with the shorter mono-unsaturated oleoyl chain (18:1-GalCer) results in an amphiphile that also forms cylindrical structures using DMF/H_2O precipitation (Fig. 5b, Table 1). In contrast, Brown found that 18:1-GalCer formed multilamellar cylindrical bilayers, sheets, and liposomes when prepared by freeze-thaw cycling (Kulkarni and Brown, 1996). Brown also studied 22:1-GalCer which formed cylindrical microstructures whereas 20:1-GalCer formed helical ribbons (Kulkarni and Brown, 1996).

The cerebroside lignoceroyl-GalCer (24:0-GalCer), which possesses the long saturated 24:0 chain formed a mixture of ribbons and liposomes using freeze-thaw cycling (Kulkarni et al., 1995). In the present study we found that stearoyl-GalCer (18:0-GalCer) and palmitoyl-GalCer (16:0-GalCer) also form twisted ribbons using the DMF/H_2O method (Fig. 5c–d, Table 1). In contrast, Curatolo reported that lyophilized 16:0-GalCer formed 100 nm diameter multilamellar tubules when suspended in water (Curatolo and Neuringer, 1986). In general, the widths of DMF/H_2O precipitated ribbons are narrower than Brown's freeze-thawed 24:0-GalCer ribbons. The lengths were also many microns but intertwining of the fibers made this difficult to accurately determine.

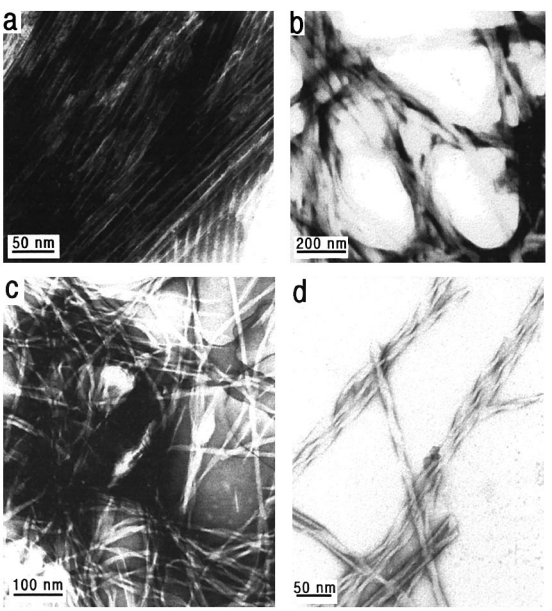

Fig. 5. Negatively stained, DMF/H_2O precipitated TEM images of: (a) 24:1-GalCer: (b) 18:1-GalCer: (c) 18:0-GalCer: (d) 16:0-GalCer. The unsaturated sphingolipids form cylindrical structures whereas the saturated lipids form ribbons.

The unsaturated fatty acyl containing GalCer, 24:1-GalCer and 18:1-GalCer, as well as NFA-GalCer and HFA-GalCer HARMs are suspected to be hollow based on negative stained TEM. A negatively stained TEM of a lipid HARM should appear uniformly dark if the object is solid whereas two parallel white lines would be prevalent against a dark background if the object is hollow. Brown's freeze-fracture electron microscopy on freeze-thaw prepared 24:1-GalCer which is of similar size to that of DMF/H_2O precipitated 24:1-GalCer was unable to unambiguously determine if the tubules were hollow or filled. The observation that some HFA-GalCer tubules terminate in a helical ribbon (upper left of Fig. 4b) supports the idea that these structures may be hollow.

Collectively, these results suggest that a mono-unsaturated fatty acyl chain GalCer is required for cylindrical tubule formation. Furthermore, the unsaturation requirement is not alleviated by shortening the acyl chain. Perhaps the *cis* double bond kink in 24:1-GalCer and 18:1-GalCer fatty acids relaxes the rigidity of the system allowing tubule formation as opposed to ribbons (reduces curvature frustration).

3.3. Pure ceramides

Despite earlier reports that ceramide containing a mixture of fatty acid chains was unable to form HARMs by thermal cycling, (Archibald and Yager, 1992) we are able to show that pure single molecular species of ceramide form HARMs using DMF/H_2O precipitation. Nervonoyl-Cer (24:1-Cer) forms cylindrical HARMs when viewed by TEM (Fig. 6a, Table 1). The density appears uniform across the diameter of the fibers suggesting that solid or cochleate cylinders are being formed. This is in contrast to the cylindrical nanostructures formed from 24:1-GalCer prepared under identical conditions (compare Fig. 5a

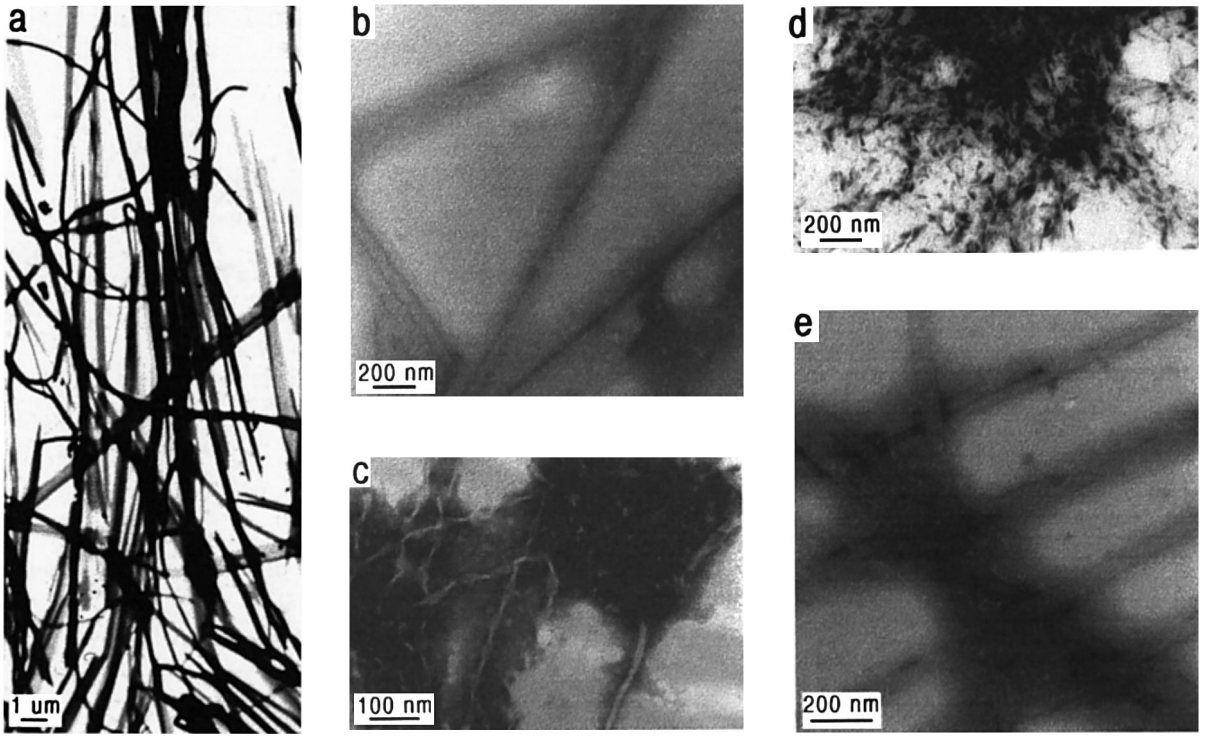

Fig. 6. TEM images of DMF/H_2O precipitated: (a) unstained 24:1-Cer; (b) negatively stained 18:1-Cer; (c) negatively stained 18:0-Cer; (d) negatively stained 16:0-Cer; (e) unstained 6:0-Cer.

and Fig. 6a). Similar dense HARMs are formed from pure oleoyl-Cer (18:1-Cer, Fig. 6b, Table 1). On the other hand, long saturated fatty acid chain-containing ceramides, stearoyl-Cer (18:0-Cer) and palmitoyl-Cer (16:0-Cer) form twisted ribbons (Fig. 6(c and d), Table 1). The short chain hexanoyl-Cer (6:0-Cer) forms cylindrical structures (Fig. 6e, Table 1), and acetyl-Cer (2:0-Cer) did not form any discernible HARMs (not shown).

These results imply that the hydrophilic galactose headgroup is not required for HARM formation, although the presence of the carbohydrate influences HARM morphology. Furthermore, the ceramide study supports the notion that the presence of saturated fatty acid chains favors twisted ribbons whereas the presence of mono-unsaturated chains favors cylindrical HARMs (except for 6:0-Cer). In the case of 6:0-Cer, the fatty acid side chain is much shorter than the sphingosine hydrocarbon chain. This high degree of chain mismatch may cause the headgroup to have a larger tilt relative to the hydrophobic alkyl region which may cause a significant packing perturbation.

3.4. Amide importance

The importance of an amide versus a secondary amine in the ceramide structure for HARM formation was studied by preparing the secondary amine formed by hydride reduction of the amide in 24:1-Cer (24:1-Amine). DMF/H_2O precipitation of 24:1-amine produced amorphous structures with no discernible HARMs (not shown). Amphiphiles that lack the fatty acid chain were also studied. Archibald and Mann reported that sphingosine formed cochleate cylinders by precipitation from water by addition of methanol (Archibald and Mann, 1993). In the present

study, DMF/H_2O precipitation of sphingosine fails to form any HARMs. On the other hand, psychosine (GalCer lacking the fatty acid chain) forms long thin HARMs when submitted to DMF/H_2O treatment (Fig. 7, Table 1). In contrast, thermal cycling of aqueous dispersions of psychosine did not lead to HARMs (Archibald and Yager, 1992). Collectively, these results suggest that two-chain sphingolipids require the presence of an amide carbonyl to form HARMs; however, a single-chain sphingolipid does not require the carbonyl for HARM formation.

3.5. Amino acid headgroups

One of the long term goals of these studies is to fabricate HARMs composed of lipidated drugs, such as lipidated peptides, for novel drug delivery strategies. In this context, we examined cerebroside-based amphiphiles that contain amino acids in place of the polar galactose headgroup. Modeling studies using proline in place of galactose suggested that microstructure formation would not be perturbed. Proline, like galactose, is cyclic, has hydrogen bond donors/acceptors and is of comparable size. Surprisingly, NAcPro-24:1-Cer did not form any discrete HARMs using DMF/H_2O precipitation, thermal or freeze-thaw cycling, or pyridine evaporation (not shown). NAcGly-24:1-Cer, containing a smaller head group, forms long thin fibers (Fig. 8). Clearly the structure of the head group greatly influences HARM morphology.

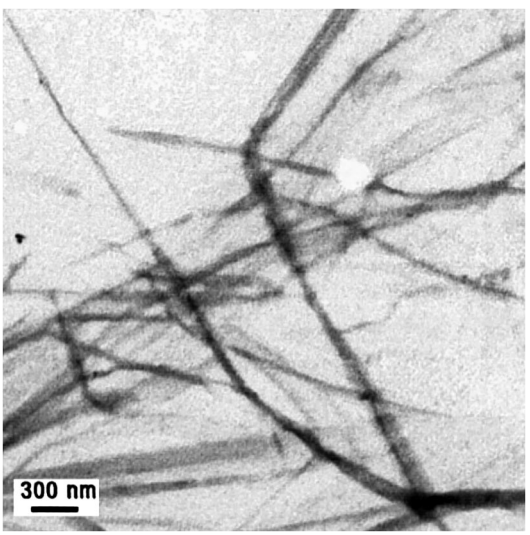

Fig. 7. TEM image of unstained, DMF/H_2O precipitated psychosine.

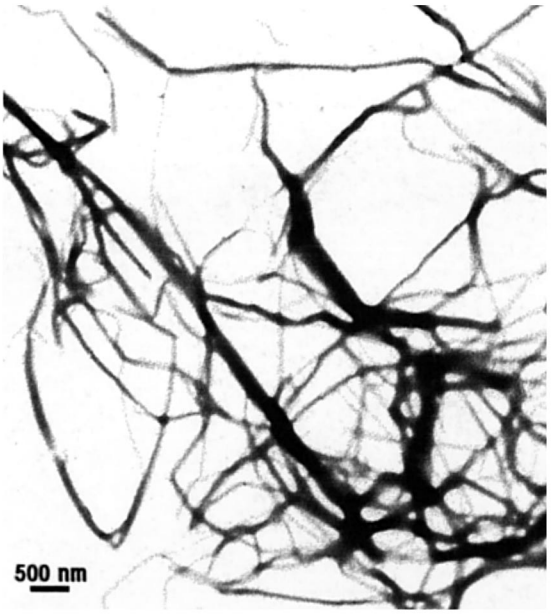

Fig. 8. TEM image of negatively stained, DMF/H_2O precipitated NAcGly-24:1-Cer.

3.6. Mixed lipid systems

Despite the inability of NAcPro-24:1-Cer to independently form discrete structures, we tried doping NAcPro-24:1-Cer into HARMs formed from 24:1-Cer, 24:1-GalCer, or NFA-GalCer (matrix). An intimate mixture of NAcPro-24:1-Cer with the HARM-forming amphiphile was first made using DMF. Water was then added to cause precipitation (Section 2). TEM images of the structures are similar to those obtained from DMF/H_2O precipitation of the pure matrix amphiphiles. The HARMs were isolated and submitted to compositional analysis by NMR (Section 2). NAcPro-24:1-Cer could be doped into either 24:1-GalCer or NFA-GalCer HARMs up to 25 mole% without affecting tubule formation. On average, the tubules were very long (micrometers)

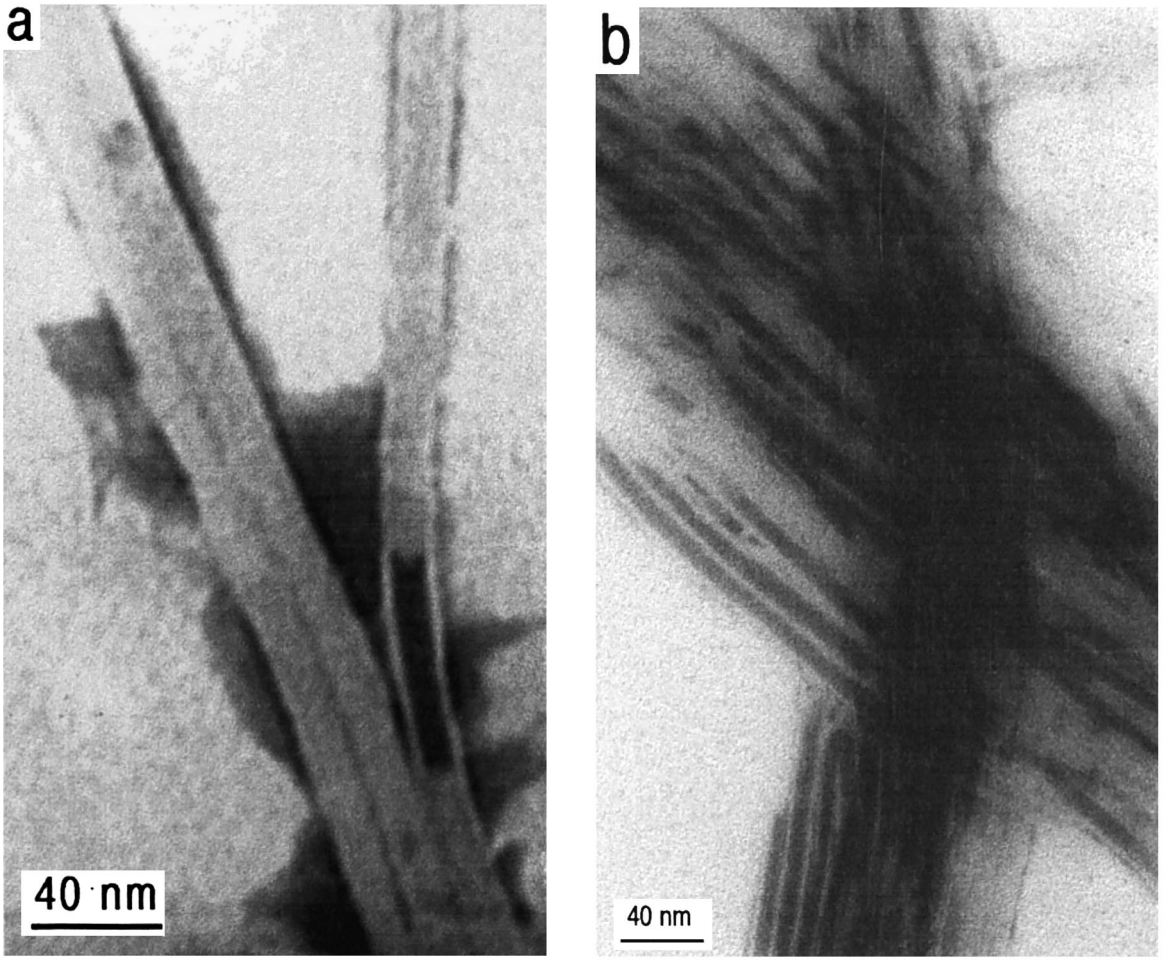

Fig. 9. TEM images of negatively stained, DMF/H_2O precipitated: (a) 1:3 NAcPro-24:1-Cer : 24:1-GalCer mixed lipid system: (b) 1:3 NAcPro-24:1-Cer : NFA-GalCer mixed lipid system.

with diameters of 13-20 nm (Fig. 9, Table 1). Increasing the NAcPro-24:1-Cer component beyond 25% resulted in the formation of vesicles in addition to tubules (not shown).

NMR analysis of the tubules formed from the 1:3 NAcPro-24:1-Cer : 24:1-GalCer or 1:3 NAcPro-24:1-Cer : NFA-GalCer mixed lipid systems retain the original solution composition. In the former case, differential scanning calorimetery was employed to determine if the lipids were ideally mixed (Fig. 10). Intimately mixed binary systems should show a single exothermic peak between the T_m of either pure component alone. As evident, the doped systems exhibit two transitions near the T_m of pure lipids implying non-ideal mixing. The presence of small transitions before and after the main NFA-GalCer transition (52 and 75°C) may be due to the presence of previously reported metastable states (Maggio et al., 1985a,b; Haas and Shipley, 1995; Curatolo, 1982). In pure NFA-GalCer, these metastable states are related to the hydration state of the sphingolipid. Furthermore, the magnitude and onset temperature of these transitions are dependent on heating and cooling rates.

Since differential scanning calorimetry indicated non-ideal mixing, a second set of experiments with NAcPro-24:1-Cer and NFA-GalCer was carried out to help determine the spatial relationship of the two lipids. Three mixing possibilities are: (1) both lipids coexist in the HARM as an intimate mixture; (2) both lipids coexist in the HARM but one lipid coats the other lipid's microstructure as it precipitates from solution; or (3) both lipids independently precipitate from solution. NFA-GalCer and NAcPro-24:1-Cer microstructures were independently formed by DMF/H_2O precipitation (Fig. 11). The cerebroside, when visualized by optical microscopy, appear as thread-like structures with occasional 'crystalline' patches whereas NAcPro-24 : 1-Cer forms only spherical aggregates. The independently precipitated species were then combined so that their concentration was identical to that of the 1:3 NAcPro-24:1-Cer : NFA-GalCer mixed lipid system. Optical micrographs of the preformed then mixed lipids show a mixture of threads with spherical aggregates, whereas intimately mixed, then precipitated lipids, show only threads. Thus possibility (3) is eliminated.

Solubility arguments may be able to distinguish between coating and coprecipitation. A more hydrophobic lipid should precipitate first upon water addition to an organic solution of mixed lipids. Although not measured, NAcPro-24:1-Cer

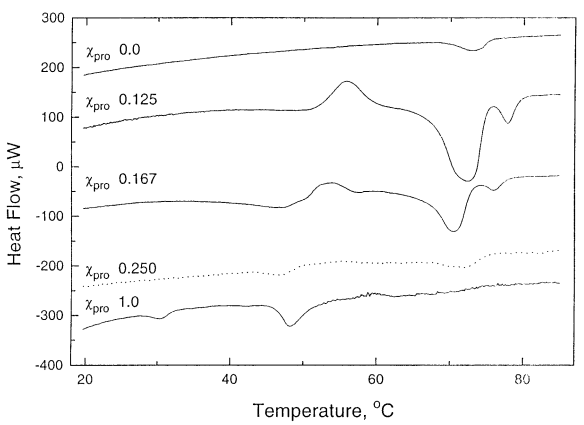

Fig. 10. Differential scanning calorimetery heating curves of NAcPro-24:1-Cer : NFA-GalCer mixed lipid systems showing that the lipids are not ideally mixed.

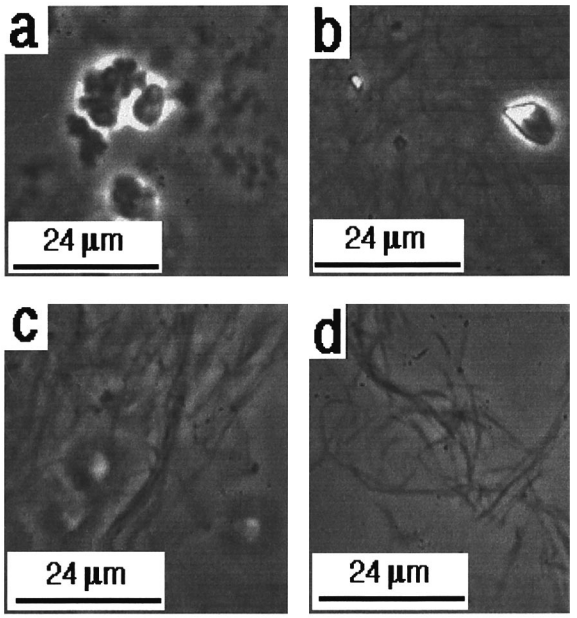

Fig. 11. Optical micrographs of DMF/H_2O precipitated: (a) NAcPro-24:1-Cer; (b) NFA-GalCer; (c) NAcPro-24:1-Cer: NFA-GalCer aggregates independently formed then mixed; (d) NAcPro-24:1-Cer : NFA-GalCer mixed and then precipitated.

should be more hydrophobic than NFA-GalCer; therefore, the amino acylated lipid should fall out of solution and form circular aggregates which would then be coated by NFA-GalCer. However, this is clearly not the case since only thread-like HARMs were seen in the mixed lipid precipitation.

In contrast, HARMs formed from NAcPro-24:1-Cer mixed into 24:1-Cer exclude the amino acid-containing amphiphile as revealed by NMR analysis and thin layer chromatography. Furthermore, optical microscopy showed that mixtures containing NAcPro-24:1-Cer in excess of 17 mole% also contained liposomes in addition to HARMs (not shown). Although it is known that sphingolipids can partition into fluid membranes to some degree, the precipitated HARMs are crystalline (Merrill et al., 1991; Maggio et al., 1985a,b). Furthermore, exclusion from the nanostructures during precipitation may be caused by significant differences in the amphiphile's aqueous solubility.

4. Conclusion

The demonstrated fact that ceramides with amino acid headgroups can independently or as a component of a binary mixture form HARMs is encouraging from the drug delivery point of view. It may be possible to attach peptides of therapeutic interest to the ceramide and still form HARMs. Due to tight crystal packing, such aggregates may be protected from proteolysis and provide a means for constant drug release via dissolution only from the ends of the HARMs. In addition, such microstructures are unlikely to be rapidly cleared from the body due to their lipophilicity, and thus may be immobilized at the desired site of action. This potentially long in vivo lifetime may make such a delivery system an ideal way to induce a strong immune response, and this may be useful for vaccine development. In mixed lipid systems, the inexpensive mixed NFA-GalCer may be used in place of the more costly single molecular galactocerebroside species. A final advantage of using such a natural tubule-forming lipid is that the human body already has enzymes that can metabolize the drug-stripped lipid core.

Acknowledgements

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