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The relationship between the structure of the headgroup of sphingolipids and their ability to form complex high axial ratio microstructures

Alex S. Goldstein [a], Michael H. Gelb [a], Paul Yager [b,*]

[a] *Departments of Chemistry and Biochemistry, University of Washington, Box 351700, Seattle, WA 98195-1700, USA*
[b] *Molecular Bioengineering Program, Department of Bioengineering, University of Washington, Box 352255, Seattle, WA 98195-2255, USA*

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Abstract

Ceramides with chemically modified polar headgroups were prepared and examined for their ability to form complex high axial ratio microstructures (CHARMS), potential drug delivery vehicles. In general, if the modified ceramide had either a hydrogen bond donor or acceptor at C-1 and C-3, including hydrophobic or hydrophilic groups attached to C-1 microstructures formed. Tolerated groups include amides, esters, sulfonates, and ethers. If modification at C-3 added significant bulk (greater than four carbons regardless of hydrophilicity), then amorphous aggregates formed. Ceramides with C-1 and C-3 bridged through a cyclic structure also made microstructures. By using a sphingolipid with an amine headgroup, CHARMs may be modified covalently after formation.

Keywords: Sphingolipid; Complex high axial ratio microstructure; Ceramide

1. Introduction

A variety of bisalkyl lipids can form complex high axial ratio microstructures (CHARMs), non-liposomal supramolecular assemblies with defined morphologies. These include diacetylenic phospholipids (Yager and Schoen, 1984; Carlson et al., 1997), dialkylglutamide (Yamada et al., 1984; Shimizu and Hato, 1993; Lee et al., 1998, 1999), perfluorinated phospholipids (Krafft et al., 1996a,b), alkylated sugars (Pinteric et al., 1973; Boettcher et al., 1996), and sphingolipids (Archibald and Yager, 1992; Archibald and Mann, 1994; Kulkarni and Brown, 1996; Kulkarni et al., 1995; Goldstein et al., 1997). In some cases, these lipids microstructures have been investigated for use as components in drug delivery systems. One requirement for such use could be covalent attachment of therapeutic agents of the surfactants. To date, sphingolipids have not been used to deliver therapeutic agents.

* Corresponding author.

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A sphingolipid consists of two divergent regions, a hydrophilic headgroup that encompasses a 1° alcohol, *N*-acylamine, and 2° allylic alcohol, and a hydrophobic region that contains the hydrocarbon chains. The ability of sphingolipids (ceramide and galactocerebroside) with varying hydrocarbon lengths and degrees of unsaturation to form CHARMs has been studied (Kulkarni and Brown, 1996; Kulkarni et al., 1995, 1999; Goldstein et al., 1997); however, studies examining chemical modification within the headgroup region are very limited in scope and depth. This paper takes a more detailed look at substitutions within the headgroup region to see which linkages may be used to tether model drug to lipid without inhibiting the formation of CHARMs. Secondly, since not all sphingolipids derivatives form microstructures, it will be shown that CHARMs may be covalently modified after formation.

2. Experimental

^{1}H NMR spectra were obtained in $CDCl_3$ using a Bruker 500 MHz (500) or Bruker 300 MHz (300) NMR spectrometer with tetramethylsilane as an internal standard. Silica gel (EM Science Silica Gel 60, 230–400 mesh) was used for all flash chromatography. Infrared (IR) spectroscopy was determined using a Perkin Elmer 1600 FTIR. Thin layer chromatography (TLC) was performed using plates coated with 250 μm Silica Gel 60 F_{254} (EM Science). All reagents were used as received. 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. In some cases, 2% aqueous ammonium molybdate (pH 5.1) was applied.

2.1. 1-Azido-24:1-Cer (1)

To 24:1-Cer (0.014 g, 21.6 μmol), triphenylphosphine (0.011 g, 43.2 μmol) in 9 ml dry CH_2Cl_2 was added crushed zinc azide bis pyridine complex (0.033 g, 108 μmol) and then diisopropylazodicarboxylate (8.5 μl, 43.2 μmol). The orange solution was stirred 75 min and then more triphenylphosphine (0.016 g, 64.8 μmol) was added. The now clear heterogeneous solution was stirred 75 min, whereupon 20 μl diisopropylazodicarboxylate was added. The solution was stirred for 1.5 h, then zinc azide bis pyridine complex (0.020 g, 64.8 μmol) was added and the solution stirred an additional 1.5 h. The mixture was absorbed onto silica gel and purified by flash chromatography (8:1–1:1 hexane:EtOAc) to provide target (0.01 g, 7%) as a clear film, R_f (1:1 hexane:EtOAc) 0.36; ^{1}H NMR (500 MHz), 5.76 (m, 1H, C-5), 5.47 (dd, 1H, C-4, $J = 3.4$, 7.7 Hz), 5.35 (t, 2H, C-15′, C-16′, $J = 7.7$ Hz), 4.20 (m, 3H, C-1, C-3), 3.96 (m, 1H, C-2), 2.17–1.95 (m, 8H, C-6, C-2′, C-14′, C-17′), 1.62 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′, $J = 6.6$ Hz); IR (neat) 3294, 2925, 2100, 1751 cm^{-1}.

2.2. 1-O-triphenylmethyl-16:0-Cer (3)

16:0-Cer (0.112 g, 208 μmol), triphenylmethyl chloride (0.064 g, 229 μmol) and DMAP (0.051 g, 416 μmol) in 20 ml dry toluene was refluxed overnight under N_2. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (9:1–2:1 hexane:EtOAc) to provide the target as a white solid (0.089 g, 55%), R_f (3:1 hexane:EtOAc) 0.32; ^{1}H NMR (500 MHz) 7.41–7.25 (15H), 6.07 (d, 1H, NH, $J = 7.4$ Hz), 5.62 (m, 1H, C-5), 5.27 (dd, 1H, C-4, $J = 6.2$, 15.5 Hz) 4.17 (m, 1H, C-2), 4.06 (m, 1H, C-3), 3.39 (dd, 1H, C-1, $J = 3.7$, 9.9 Hz), 3.5 (m, 2H, C-1), 2.20 (t, 2H, C-2′, $J = 6.8$ Hz), 1.92 (m, 2H, C-6), 1.64 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-16′, $J = 6.8$ Hz).

2.3. 1-O-triphenylmethyl-8:0-Cer (4)

8:0-Cer (0.132 g, 310 μmol), triphenylmethyl chloride (0.086 g, 310 μmol) and DMAP (0.038 g, 310 μmol) in 50 ml toluene was refluxed overnight. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (8:1–2:1 hexane:EtOAc) to the target as a white solid (0.116 g, 56%), R_f (3:1 hexane:EtOAc) 0.23; ^{1}H NMR (500 MHz) 7.41–7.25 (15H), 6.06 (d, 1H, NH, $J = 8.0$ Hz), 5.62 (m,

1H, C-5), 5.26 (dd, 1H, C-4, $J = 6.2$, 15.5 Hz), 4.17 (m, 1H, C-2), 4.07 (dd, 1H, C-3, $J = 3.7$, 8.0 Hz), 3.39 (dd, 1H, C-1, $J = 3.7$, 9.9 Hz), 3.30 (39 (dd, 1H, C-1, $J = 3.7$, 9.9 Hz), 2.20 (t, 2H, C-2′, $J = 7.5$ Hz), 1.92 (m, 2H, C-6), 1.64 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-8′, $J = 6.8$ Hz).

2.4. 1-O-(4,4′-dimethoxytriphenylmethyl)-24:1-Cer (5)

To 24:1-Cer (0.031 g, 47.8 μmol), and DMAP (0.006, 52.6 μmol) in 10 ml toluene was added 4,4′-dimethoxytriphenylmethyl chloride (0.018 g, 52.6 μmol). The reaction was refluxed for 21 h and the solvent removed by rotary evaporation. The yellow residue was purified by flash chromatography (8:1–2:1 hexane:EtOAc) to provide the target as a white solid (0.010 g, 23%), R_f (3:1 hexane:EtOAc) 0.14; ^{1}H NMR (500 MHz) 7.39–6.82 (m, 13H), 6.05 (d, 1H, NH), 5.62 (dt, 1H, C-5), 5.35 (t, 2H, C-15′, C-16′), 5.29 (dd, 1H, C-4), 4.18 (bt, 1H, C-3), 3.79 (s, 6H, OCH_3), 3.42 (d, 1H, OH), 3.41 (dd, 1H, C-1) 3.31 (dd, 1H, C-1), 2.19 (t, 2H, C-2′), 2.01 (m, 4H, C-14′, C-17′). 1.92 (m, 2H, C-6), 1.59 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′).

2.5. 1-O-allyl-24:1-Cer (6) and 3-O-allyl-24:1-Cer (18)

To 24:1-ceramide (0.029 g, 45 μmol) in 10 ml benzene was added powdered sodium hydroxide (0.065 g, 1.5 mmol) allyl bromide (90 μl, 1 mmol). The reaction was heated to reflux for 18.5 h. After cooling to room temperature, the solvent was removed under reduced pressure and the residue purified by flash chromatography (7:1–0:1 hexane:EtOAc) to provide 3-*O*-allyl-24:1-Cer (0.010 g, 32%) and 1-*O*-allyl-24:1-Cer (0.009 g, 29%) as white films.

2.5.1. 1-O-allyl-24:1-Cer (6)

R_f (1:1 hexane:EtOAc) 0.58; ^{1}H NMR (500 MHz) 6.22 (d, 1H, NH, $J = 8.0$ Hz), 5.86 (m, 1H, CH=CH_2), 5.76 (dt, 1H, C-5), 5.49 (dd, 1H, C-4 $J = 9.3$, 15.5 Hz), 5.35 (t, 2H, C-15′, C-16′, $J = 4.3$ Hz), 5.24 (dd, 2H, CH=CH_2), 4.16 (bs, 1H), 4.05 (bs, 1H), 3.96 (t, 2H, OCH_2, $J = 6.2$ Hz), 3.75 (dd, 1H, C-1, $J = 3.7$, 9.9 Hz), 3.57 (75 (dd, 1H, C-1, $J = 3.7$, 9.9 Hz) 3.41 (d, 1H, OH, $J = 8.0$ Hz), 2.22 (t, 2H, C-2′, $J = 7.4$ Hz), 2.00 (m, 6H, C-6, C-14′, C-17′), 1.63 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′, $J = 6.8$ Hz).

2.5.2. 3-O-allyl-24:1-Cer (18)

R_f (1:1 hexane:EtOAc) 0.42; ^{1}H NMR (300 MHz) 6.16 (d, 1H, NH, $J = 7.3$ Hz), 5.80 (m, 2H, C-5, CH=CH_2), 5.28 (t, 2H, C-15′, C-16′, $J = 4.5$ Hz), 5.12 (t, 2H, CH=CH_2), 3.90 (m, 4H, C-2, C-1, OCH_2), 3.72 (dd, 1H, C-1, $J = 5.9$, 12.7 Hz), 3.52 (bd, 1H, C-3, $J = 9.8$ Hz), 2.93 (bs, 1H, OH), 2.15 (t, 2H, C-2′, $J = 6.8$ Hz), 2.00 (m, 6H, C-6, C-14′, C-17′), 1.53 (m, 2H, C-3′), 0.81 (t, 6H, C-18, C-24′, $J = 6.8$ Hz).

2.6. 1-O-allyl-16:0-Cer (7) and -1-O-allyl-16:0-Cer (19)

To 16:0-Cer (0.057 g, 106 μmol) in 9 mL benzene was added powdered sodium hydroxide (0.051 g, 1.3 mmol), allyl bromide (183 μL, 2.1 mmol). The reaction was heated to reflux for 15 h. After cooling to room temperature, the solvent was removed under reduced pressure and the residue purified by flash chromatography (8:1-0:1 hexane:EtOAc) to provide 3-O-allyl-16:0-Cer (0.024 g, 39%) and 1-O-allyl-16:0-Cer (0.021 g, 34%) as white films.

3-O-allyl-16:0-Cer (7): R_f (1:1 hexane:EtOAc) 0.45; ^{1}H NMR (300 MHz) 6.27 (d, 1H, NH, J = 7.3 Hz), 5.80 (m, 2H, C-5, CH = CH_2), 5.38 (dd, 1H, C-4, J = 7.8, 15.6 Hz), 5.12 (t, 2H, CH = CH_2), 4.0 (m, 4H, C-2 C-1, OCH_2), 3.72 (dd, 1H, C-1, J = 5.9, 12.7 Hz), 3.72 (bd, 1H, C-3, J = 13.7 Hz), 3.04 (bs, 1H, OH), 2.22 (t, 2H, C-2′, J = 7.3 Hz), 2.05 (t, 2H, C-6, J = 6.8 Hz), 1.62 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-16′, J = 6.8 Hz). 1-O-allyl-16:0-Cer (19): R_f (1:1 hexane:EtOAc) 0.62; ^{1}H NMR (300 MHz) 6.18 (d, 1H, NH, J = 7.8 Hz), 5.86 (m, 2H, C-5, CH = CH_2), 5.46 (dd, 1H, C-4, J = 5.4, 15.6 Hz), 5.18 (dd, 2H, CH = CH_2), 4.17 (bs, 1H), 4.01 (bs, 1H), 3.93 (bs, 2H, OCH_2), 3.72 (dd, 1H, C-1, J = 3.4, 9.8 Hz), 3.52 (75 (dd, 1H, C-1, J = 3.4, 9.8 Hz) 3.38 (d, 1H, OH, J = 8.8 Hz), 2.19 (t, 2H, C-2′, J = 7.3 Hz), 2.01 (m, 2H, C-6), 1.60 (m, 2H, C-3′), 0.85 (t, 6H, C-18, C-16′, J = 6.8 Hz).

2.7. 1-*O*-*t*-butyldiphenylsilyl-24:1-Cer (9)

To 3-*O*-tBDPS-24:1-Cer (0.032 g, 36 μmol) in 3 ml dry THF was added NaH (0.001 g, 40 μmol). After stirring for 10 min, 2-bromoethanol 3.1 μl, 43 μmol) was added and the reaction stirred for 3 h. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (10:1–0:1 hexane:EtOAc) to provide in addition to starting material some target as a clear film (0.007 g, 21%) R_f (3:1 hexane:EtOAc) 0.31; ^{1}H NMR (500 MHz) 7.70–7.32 (m, 10H), 6.14 (d, 1H, NH, $J = 7.4$ Hz), 5.83 (dt, 1H, C-5), 5.54 (dd, 1H, C-4), 5,41 (t, 2H, C-15′, C-16′), 4.02 (m, 2H, C-1, C-3), 3.81 (bd, 1H, C-1), 3.58 (d, 1H, OH), 2.25 (t, 2H, C-2′), 2.07 (m, 6H, C-6, C-14′, C-17′), 1.66 (m, 2H, C-3′), 1.14 (s, 9H), 0.95 (t, 6H, C-18, C-24′, $J = 6.2$ Hz).

2.8. 1-*O*-(2-napthoic acid)-24:1-Cer (10)

To 3-*O*-tBDPS-24:1-Cer (0.063 g, 71.1 μmol), 2-napthoic acid (0.013 g, 78.2 μmol) and DMAP (0.010 g, 78.2 μmol) in 10 ml dry 1:1 CH_3CN:CH_2Cl_2 was added dicyclohexylcarbodiimide (0.016 g, 78.2 μmol). The reaction was stirred for 22 h and the white precipitate removed by filtration. The solvent was evaporated and the residue purified by flash chromatography (8:1–2:1 hexane:EtOAc) to provide 1-*O*-(2-napthoyl)-3-*O*-tBDPS-24:1-Cer (0.052 g, 70%, as a white residue: R_f (3:1 hexane:EtOAc) 0.57; ^{1}H NMR (500 MHz) 7.98–7.29 (m, 17H), 5.57 (d, 1H, NH), 5.50 (dd, 1H, C-4), 5.47 (dt, 1H, C-5), 5.35 (t, 2H, C-15′, C-16′), 4.66 (dd, 1H, C-1), 4.50 (dd, 1H, C-1), 4.46 (m, 1H, C-2), 4.38 (bt, 1H, C-3), 2.03–1.83 (m, 8H, C-6, C-2′, C-14′, C-17′), 1,41 (m, 2H, C-3′). 0.88 (t, 6H, C-18, C-24′).

To 1-*O*-(2-napthoyl)-3-*O*-tBDPS-24:1-Cer (0.051 g, 49 μmol) in 15 ml dry THF was added 1.0 M tetrabutylammonium fluoride (14 μl). The reaction was stirred 3 h, whereupon the solvent was removed by rotary evaporation and the residue purified by flash chromatography (6:1–2:1 hexane:EtOAc) to provide the title compound (0.026 g, 67%) as a clear residue, R_f (1:1 hexane:EtOAc) 0.63; ^{1}H NMR (500 MHz) 8.59 (bs, 1H), 8.03 (dd, 1H), 7.95 (d, 1H), 7.87 (d, 2H), 7.57 (m, 2H), 6.04 (d, 1H, NH), 5.79 (dt, 1H, C-5), 5.55 (dd, 1H, C-4), 5.35 (t, 2H, C-15′, C-16′), 4.63 (dd, 1H, C-1), 4.46 (m, 2H, C-1, C-3), 4.31 (bs, 1H, C-2), 2.97 (bs, 1H, OH), 2.19 (t, 2H, C-2′), 2.01 (m, 6H, C-6, C-14′, C-17′), 1.59 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′).

2.9. 1-Biotinamido-24:1-Cer (11)

To 1-phthalimido-3-*O*-tBDPS-24:1-Cer (intermediate in synthesis of compound 15, 0.046 g, 45.3 μmol) in 12 ml 95% EtOH was added 0.23 ml hydrazine hydrate. The solution was heated to reflux for 2 h, then the solvent removed by rotary evaporation, and the residue purified by flash chromatography (1:0–9:1 EtOAc:MeOH) to give 1-amino-3-*O*-tBDPS-24:1-Cer (0.036 g, 90%) as a clear film, R_f (MeOH) 0.48; ^{1}H NMR (500 MHz) 7.77–7.34, (m, 10H) 6.02 (d, 1H, amide), 5.34 (m, 4H, C-4, C-5, C-15′, C-16′), 4.25 (m, 1H, C-3), 3.99 (m, 1H, C-2), 3.02 (bs, 2H, C-1), 2.55 (bs, 2H, NH_2) 2.02–1.83 (m, 8H, C-6, C-2′, C-14′, C-17′), 1.05 (s, 9H, *t*-Bu), 0.88 (t, 6H, C-18, C-24′ $J = 7.2$ Hz).

To 1-amino-3-*O*-tBDPS-24:1-Cer (0.021 g, 23.7 μmol) in 12 ml dry THF was added 18 μl of 1.0 M (in THF) tetrabutylammonium fluoride. The reaction stirred 3 h, the solvent removed by rotary evaporation, and the residue purified by flash chromatography (1:0–0:1 EtOAc:MeOH). Fractions containing 1-amino-24:1-Cer were combined, diluted with H_2O, extracted with $5 \times Et_2O$, and the extracts concentrated to provide the desired material as a clear film (0.013 g, 87%), R_f (1:1 hexane:EtOAc) 0.36; ^{1}H NMR (500 MHz), 5.76 (m, 1H, C-5), 5.47 (dd, 1H, C-4, $J = 3.4$, 7.7 Hz), 5.35 (t, 2H, C-15′, C-16′, $J = 7.7$ Hz), 4.20 (m, 3H, C-1, C-3), 3.96 (m, 1H, C-2), 2.17–1.95 (m, 8H, C-6, C-2′, C-14′, C-17′), 1.62 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′ $J = 6.6$ Hz); IR (neat) 3294, 2925, 2100, 1751 cm^{-1}.

To 1-amino-24:1-Cer (0.005 g, 7 μmol) in 5 ml dry DMF was added biotin-tetrafluorophenyl ester (0.006 g, 16 μmol). After stirring overnight, the solvent evaporated in vacuo. Flash chromatography of the residue (9:1 $CHCl_3$:MeOH) provided the target as a clear oil (0.001 g, 14%), R_f (5:1 $CHCl_3$:MeOH) 0.79; ^{1}H NMR (500 MHz)

7.01 (m, 1H, NH), 6.38 (s, 1H, NH) 6.25 (s, 1H, NH), 5.77 (m, 1H, C-5), 5.49 (dd, 1H, C-4), 5.35 (m, 2H, C-15′, C-16′), 4.51 (s, 1H, biotin bridgehead), 4.34 (s, 1H, biotin bridgehead), 4.11 (m, 1H, C-3), 3.37 (m, 1H, C-2), 3.29 (m, 3H, C-1, biotin methine), 2.91 (m, 1H, biotin methylene), 2.74 (m, 1H, biotin methylene), 2.33 (m, 2H, a-biotin amide), 1.25 (m, 4H, biotin), 1.47 (m, 2H, d-biotin amide), 0.88 (t, 6H, C-18, C-24′ $J = 6.8$ Hz).

2.10. 1-Bromoacetamido-24:-1-Cer (12)

To 1-bromoacetamido-3-*O*-tBDPS-24:1-Cer (0.003 g, 3 μmol) in 3 ml THF was added 25 μl of 3% HCl/MeOH (v:v). The solution was stirred for 22 h whereupon 10 ml Et_2O was added. The mixture was washed with 3 ml 5% $NaHCO_3$ (aq) and 5 ml H_2O. The solvent was removed under vacuum and the residue purified by flash chromatography (1:1–0:1 hexane:EtOAc) to provide the desired material as a clear film (0.001 g, 50%), R_f (1:1 hexane:EtOAc) 0.19; ^{1}H NMR (500 MHz) 6.06 (d, 1H, NH, $J = 6.8$ Hz), 5.77 (dt, 1H, C-5), 5.49 (dd, 1H, C-4), 5.35 (t, 2H, C-15′, C-16′, $J = 4.9$ Hz), 4.18 (1H, C-3), 4.06 (1H, C-2), 3.65 (1H), 3.50 (m, 2H), 3.32 (m, 1H), 2.75 (m, 1H), 2.20–1.98 (m, 8H, C-6, C-2′, C-14′, C-17′), 0.88 (t, 6H, C-18, C-24′); ES-MS 769 (M +, 72%).

2.11. 1-Hydroxyacetamido-3-O-t-butyldiphenylsilyl-24:1-Cer (13)

To 3-*O*-tBDPS-1-bromoacetamido-24:1-Cer (0.109 g, 108 μmol) in 20 ml *N*-methyl-2-pyrrolidone was added water (0.3 ml, 17 mmol) and $NaHCO_3$ (0.009 g, 108 μmol). The reaction was incubated under dry N_2 (g) at 93°C for 18.5 h. After cooling to room temperature, 20 ml H_2O was added and the solution was extracted twice with 20 ml Et_2O. The organic layers were washed with 15 ml H_2O, 15 ml saturated NaCl (aq), and 15 ml H_2O. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (3:1–0:1 hexane:EtOAc to provide the desired material as a clear film (0.073 g, 72%), R_f (EtOAc) 0.54; ^{1}H NMR (500 MHz) 7.68–7.37 (m, 10H), 6.81 (s, 1H, AcNH), 5.74 (d, 1H, NH, $J = 8.7$ Hz), 5.47–5.40 (m, 2H, C-4, C-5), 5.35 (t, 2H, C-15′, C-16′, $J = 4.3$ Hz), 4.24 (1H, C-3), 3.99 (s, 2H, $HOCH_2C(O)$), 3.95 (m, 1H, C-2), 3.50 (m, 2H, C-1), 2.65 (bt, 1H, HOAc), 2.02–1.84 (m, 8H, C-6, C-2′, C-14′, C-17′), 1.44 (m, 2H, C-3′), 1.08 (s, 9H, t-Bu), 0.88 (t, 6H, C-18, C-24′ $J = 6.2$ Hz); FAB-MS 944 (M^+, 3%), 886 (M-t-Bu, 15%) 866 (M-Ph, 6%), 687 (M-tBDPSiOH, 47%).

2.12. 1-Hydroxyacetamido-24:1-Cer (14)

To 1-bromoacetamido-3-*O*-tBDPS-24:1-Cer (0.010 g, 10 μmol) in 2 ml *N*-methyl-2-pyrrolidone was added water (0.3 ml, 17 mmol) and $NaHCO_3$ (0.003 g, 30 μmol). The reaction was incubated under dry N_2 (g) at 105°C for 23.5 h. After cooling to room temperature, 10 ml H_2O was added and the solution was extracted twice with 10 ml Et_2O. The organic layers were washed with 10 ml H_2O, 10 ml saturated NaCl (aq), and 10 ml H_2O. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (3:1–0:1 hexane:EtOAc) to provide the desired material as a clear film (0.002 g, 20%), R_f (EtOAc) 0.07; ^{1}H NMR (500 MHz) 7.05 (s, 1H, AcNH), 6.28 (d, 1H, NH, $J = 7.4$ Hz), 5.81 (m, 1H, C-5), 5.55 (dd, 1H, C-4), 5.40 (t, 2H, C-15′, C-16′, $J = 4.3$ Hz), 4.15 (m, 4H, C-2, C-3, HOCH2), 3.67 (m, 1H, C-1), 3.50 (m, 1H, C-1), 3.16 (bs, 1H, OH), 2.80 (bs, 1H, OH) 2.24–1.84 (m, 8H, C-6, C-2′, C-14′, C-17′), 0.88 (t, 6H, C-18, C-24′ $J = 6.2$ Hz); FAB-MS 706 (M^+, 6%), 688 (M-H_2O, 12%).

2.13. 1-Phthalimido-24:1-Cer (15)

To-3-*O*-tBDPS-24:1-Cer (0.111 g, 125 μmol), triphenylphosphine (0.164 g, 626 μmol), and phthalimide (0.020 g, 138 μmol) in 20 ml dry THF was added diisopropylazodicarboxylate (27 μl, 138 μmol). The initially orange solution was stirred for 3 h. The solvent was evaporated and the residue purified by flash chromatography (15:1–5:1 hexane:EtOAc) to provide 1-phthalimido-3-*O*-tBDPS-24:1-Cer as a white solid (0.108 g, 85%), R_f (3:1 hexane:EtOAc) 0.65; ^{1}H NMR (500 MHz) 7.81–7.34 (m, 14H), 5.62 (d, 1H, NH), 5.52 (m, 2H, C-4, C-5), 5.35 (t, 2H, C-15′, C-16′), 4.29 (m, 2H, C-2, C-3), 3.96 (dd, 2H, C-1), 1.99 (m, 8H, C-6, C-2′, C-14′, C-17′), 1.60 (m, 2H, C-3′), 1.11 (s, 9H, *t*-BuSi), 0.88 (t, 6H, C-18, C-24′).

To 1-phthalimido-3-*O*-tBDPS-24:1-Cer (0.037 g, 36.4 μmol) in 12 ml dry THF was added 1.0 M (in THF) tetrabutylammonium fluoride (11.6 μl, 40.1 μmol). The solution was stirred for 4 h. The solvent was evaporated and the residue purified by flash chromatography (6:1–1:1 hexane:EtOAc) to provide the title material as a white solid (0.007 g, 25%), R_f (3:1 hexane:EtOAc) 0.09; ^{1}H NMR (500 MHz) 7.88–7.71 (m, 4H), 6.15 (d, 1H, NH), 5.75 (dt, 1H, C-5), 5.50 (dd, 1H, C-4), 535 (t, 2H, C-15′, C-16′), 4.35 (m, 1H, C-2), 4.23 (bs, 1H, C-3), 3.87 (dd, 2H, C-1), 3.07 (bs, 1H, OH), 2.11 (m, 2H, C-2′), 2.02 (m, 6H, C-6, C-14′, C-15′), 1.50 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′).

2.14. 3-Keto-24:1-Cer (16)

To 24:1-Cer (0.012 g, 18.5 μmol) in 20 ml dry benzene was refluxed with manganese(IV) oxide (0.012 g, 122.4 μmol) for 5 h and then stirred an additional 12 h at room temperature. The solvent was removed by rotary evaporation and the residue was purified by flash chromatography (5:1–2:1 hexane:EtOAc) to provide desired material (0.005 g, 42%) as a white film, R_f (1:1 hexane:EtOAc) 0.45; ^{1}H NMR (500 MHz) 7.10 (dt, 1H, C-5), 6.77 (bs, 1H, amide), 6.26 (d, 1H, C-4), 5.35 (t, 2H, C-15′, C-16′), 4.90 (m, 1H, C-2), 3.89 dd, 2H, C-1), 3.31 (bs, 1H, OH), 2.27 (m, 4H, C-2′, C-6), 2.01 (m, 4H, C-14′, C-17′), 1.65 (m, 1H, C-3′), 1.46 (m, 1H, C-3′), 0.88 (t, 6H, C-18, C-24′).

2.15. 3-Keto-24:1-Cer (17)

16:0-Cer (0.070 g, 0.13 mmol) and DDQ (0.295 g, 1.30 mmol) were refluxed for 5 h in dry benzene and then stirred at 20°C for 4 days. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (6:1–2:1 hexane:EtOAc) to provide the desired material as a red solid. This red solid was dissolved in 10 ml Et_2O and washed with 8×5 ml H_2O, 5 ml brine, 5 ml saturated $NaHCO_3$ (aq) and 3×5 ml H_2O. The gold colored ether layer was filtered through celite, evaporated and the residue purified by flash chromatography (3:1–1:1 hexane:EtOAc) to provide the title compound as a white solid (0.010 g, 14%), R_f (1:1 hexane:EtOAc) 0.26; ^{1}H NMR (500 MHz) 7.08 (m, 1H, C-5), 6.70 (1H, NH), 6.26 (d, 1H, C-4, $J = 17.3$ Hz), 4.88 (1H, C-2), 3.94 (1H, C-1), 3.79 (1H, C-1), 3.27 (1H, OH), 2.25 (m, 4H, C-6, C-2′), 0.87 (t, 6H, C-16′, C-18).

2.16. 1-Phthalimido-3-keto-24:1-Cer (18)

To 3-keto-24:1-Cer (0.005 g, 7.7 μmol), triphenylphosphine (0.010 g, 38.7 μmol), and phthalimide (0.001 g, 8.5 μmol) in 1 ml dry THF was added diisopropylazodicarboxylate (0.002 g, 1.7 μl). The reaction was stirred overnight. The solvent was removed by rotary evaporation and the residue was purified by flash chromatography (6:1–3:1 hexane:EtOAc) to give the product (0.002 g, 33%) as a white film, R_f (3:1 hexane:EtOAc) 0.26; ^{1}H NMR (500 MHz) 7.90–7.76 (m, 4H), 6.41 (s, 1H, NH), 5.71 (dt, 1H, C-5), 5.54 (d, 1H, C-4), 5.35 (t, 2H, C-15′, C-16′), 5.11 (d, 2H, C-1), 4.97 (t, 1H, C-2), 2.39 (t, 2H, C-2′), 2.02 (m, 6H, C-6, C-14′, C-17′), 1.66 (m, 1H, C-3′), 0.88 (t, 6H, C-18, C-24′).

2.17. 1-O-triphenylmethyl-3-O-methoxymethyl-24:1-Cer (21)

To 1-*O*-triphenylmethyl-24:1-Cer (0.040 g, 45 μmol) and diisopropylethylamine (1 ml) in 25 ml dry THF was added bromomethylmethyl, ether (16 μl, 195 μmol). The reaction was stirred overnight and the solvent removed by rotary evaporation. The residue was purified by flash chromatography (8:1–0:1 hexane:EtOAc) to provide starting material and pure target as a clear film (0.016 g, 38%), R_f (3:1 hexane:EtOAc) 0.58; ^{1}H NMR (500 MHz) 7.44–7.22 (m, 15H), 5.67 (m, 2H, NH, C-5), 5.35 (t, 2H, C-15′, C-16′, $J = 4.3$ Hz), 5.23 (dd, 1H, C-4, $J = 8.0$, 15.5 Hz), 4.62 (d, 1H, OCH_2O, $J = 6.8$ Hz), 4.46 (d, 1H, OCH_2O, $J = 6.8$ Hz), 4.25 (m, 1H, C-2), 4.19 (t, 1H, C-3, $J = 6.8$ Hz), 3.38 (dd, 1H, C-1, $J = 5.0$, 9.9 Hz), 3.25 (dd, 1H, C-1, $J = 5.0$, 9.9 Hz), 3.21 (s, 3H, OCH_3), 2.10 (t, 2H, C-2′, $J = 6.8$), 2.00 (m, 6H, C-6, C-14′, C-17′), 1.67 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′, $J = 6.2$ Hz).

2.18. 3-O-methoxymethyl-24:1-Cer (22)

To 1-*O*-triphenylmethyl-3-*O*-methoxymethyl-24:1-Cer (0.016 g, 17 μmol) in 10 ml 1:1

CH_2Cl_2:MeOH was added *p*-toluenesulfonic acid monohydrate (0.008 mg, 42 μmol). The reaction was stirred overnight and then reduced to 25% of the initial volume by rotary evaporation. The solution was diluted with 25 ml Et_2O and washed with 20 ml saturated $NaHCO_3$ (aq) and 20 ml H_2O. The organic layer was evaporated and the residue purified by flash chromatography (5:1–0:1 hexane:EtOAc) to provide the target as a white film (0.010 g, 83%), R_f (1:1 hexane:EtOAc) 0.23; ^{1}H NMR (500 MHz) 6.23 (d, 1H, NH, $J = 7.4$ Hz), 5.75 (m, 1H, C-5), 5.35 (m, 3H, C-4, C-15′, C-16′), 4.60 (d, 1H, OCH_2O, $J = 6.8$ Hz), 4.54 (d, 1H, OCH_2O, $J = 6.8$ Hz), 4.21 (m, 1H, C-3), 3.96 (m, 2H, C-1), 3.66 (m, 1H, C-2), 3.38 (s, 3H, OCH_3), 2.21 (m, 2H, C-2′), 2.05 (m, 6H, C-6, C-14′, C-17′), 1.62 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24′, $J = 6.8$ Hz).

2.19. 3-*O*-methoxyethoxymethyl-24:1-Cer (23)

To 1-*O*-tBDPS-24:1-Cer (0.025 g, 28 μmol) in 15 ml dry CH_2Cl_2 was added diisopropylethylamine (9.8 μl g, 56 μmol) and 2-methoxyethoxymethyl chloride (3.5 μl, 31 μmol). The reaction was stirred for 4 h after which additional DIPEA (0.50 ml) and MEMCI (0.25 ml) was added to the reaction stirred overnight. The solution was washed with 30 ml H_2O and 30 ml saturated NaCl (aq). The solvent was removed by rotary evaporation. The residue was dissolved in 5 ml THF and stirred with 0.25 ml 1.0 M tBAF for 2 h. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (3:1–0:1 hexane:EtOAc) to give target as a clear film (0.016 g, 76%): (EtOAc) 0.41; ^{1}H NMR (500 MHz) 6.06 (d, 1H, NH, $J = 8.0$ Hz), 5.64 (dt, 1H, C-5), 5.27 (m, 3H, C-4, C-15′, C-16′), 4.66 (d, 1H, OCH_2O, $J = 6.8$ Hz), 4.51 (d, 1H, OCH_2O, $J = 6.8$ Hz), 4.07 (t, 1H, C-3, $J = 7.4$ Hz), 3.92 (m, 2H, C-1), 3.79 (bt, 1H, C-2, $J = 8.7$), 3.55 (m, 4H, OCH_2 CH_2O), 3.35 (s, 3H, OCH_3), 2.08 (t, 2H, C-2′), 1.95 (m, 6H, C-6, C-14′, C-17′), 1.54 (m, 2H, C-3′), 0.81 (t, 6H, C-18, C-24′, $J = 6.2$ Hz).

2.20. (1,3-*O*-formyl acetal)-24:1-Cer (24)

To 3-*O*-methoxymethyl-24:1-Cer (0.036 g, 52 μmol) in 10 ml *N*-methyl-2-pyrrolidone was added 1,2-dibromoethane (0.5 ml). The reaction was incubated at 97°C for 17 h under inert atmosphere. After cooling to room temperature, 30 ml H_2O was added and the solution extracted with 2×20 ml Et_2O. The ethereal solution was washed with 20 ml H_2O, 10 ml saturated NaCl (aq) and 10 ml H_2O, and then concentrated. The residue was purified by flash chromatography (8:1–0:1 hexane:EtOAc) to give the target as a white film (0.005 g, 15%), R_f (1:1 hexane:EtOAc) 0.52; ^{1}H NMR (500 MHz) 5.80 (dt, 1H, C-5), 5.55 (m, 2H, C-4, NH), 5.38 (t, 2H, C-15′, C-16′), 5.05 (d, 1H, OCH_2O) 4.65 (d, 1H, OCH_2O), 4.54 (d, 1H, OCH_2O, $J = 6.8$ Hz), 4.18 (dd, 1H, C-3), 3.96 (m, 2H, C-1), 3.45 (m, 1H, C-2), 2.16 (m, 2H, C-2′), 2.05 (m, 6H, C-6, C-14′, C-17′), 1.62 (m, 2H, C-3′), 0.88 (t, 6H, C-18, C-24).

2.21. (1,3-*O*-hexyl acetal)-24:1-Cer (25)

To 24:1-Cer (0.005 g, 8 μmol) in 15 ml CH_2Cl_2 was added hexanal (2 μl, 15 μmol) and *p*-toluenesulfonic acid monohydrate (0.0003 g, 1 μmol). The reaction was heated to reflux overnight under inert atmosphere. After cooling to room temperature, the solvent was removed under reduced pressure and the residue purified by flash chromatography (8:1–3:1 hexane:EtOAc) to provide target as a white film (0.002 g, 33%), R_f (3:1 hexane:EtOAc) 0.13; ^{1}H NMR (500 MHz) 5.77 (dt, 1H, C-5), 5.45 (dd, 1H, C-4), 5.35 (t, 2H, C-15′, C-16′), 4.91 (d, 1H, NH), 4.52 (t, 1H, acetal), 4.22 (dd, 1H, C-3), 3.87 (m, 1H, C-2), 3.78 (t, 1H, C-1), 3.35 (t, 1H, C-1), 2.16 (m, 2H, C-2′), 2.05 (m, 6H, C-6, C-14′, C-17′), 1.64 (m, 2H, C-3′), 0.87 (t, 6H, C-18, C-24′).

2.22. (1,3-*O*-(3′hydroxy)-propyl acetal)-24:1-Cer (26)

To 24:1-Cer (0.042 g, 65 μmol) in 10 ml CH_2Cl_2 was added 3-*O*-triphenylmethyl-propanal (0.019 mg, 71 μmol) and *p*-toluenesulfonic acid monohydrate (0.012 mg, 65 μmol). The reaction was heated to reflux for 16 h. After cooling to room temperature, the reaction was washed with 10 ml saturated $NaHCO_3$ (aq) and 10 ml H_2O. The solvent was removed under reduced pressure and the residue purified by flash chromatography (4:1–0:1 hexane:EtOAc) to provide (1,3-*O*-(3′-hydroxy)-propyl ace-

tal)-24:1-Cer as a white film (0.014 g, 30%), R_f (EtOAc) 0.57; ^{1}H NMR (500 MHz) 5.76 (dt, 1H, C-5), 5.44 (dd, 1H, C-4), 5.35 (t, 2H, C-15′, C-16′), 4.92 (d, 1H, NH), 4.78 (t, 1H, acetal), 4.26 (dd, 1H, C-3), 3.92 (m, 1H, C-2), 3.78 (bd, 2H, C-1), 3.37 (t, 1H, OH), 2.10 (m, 2H, C-2′), 2.01 (m, 4H, C-14′, C-17′), 1.93 (M, 2H, C-6), 0.88 (t, 6H, C-18, C-24′, $J = 6.8$ Hz).

3. Conjugation to preformed CHARMs

To 6.5 mg of NFA-GalCer CHARMs (nonhydroxy fatty acid containing portion of galactocerebroside) in 200 μl 0.15 M $NaHCO_3$ (aq) was stirred for 96 h in the dark with 1.0 mg BODIPY-FL-NSSE (Molecular Probes). The assemblies were pelleted by centrifugation (10 min, 10 000 × *g*) and the supernatant removed. The pellet was washed with 4 × 1 ml H_2O and 2 × 1 ml 1 M NaCl (aq) with centrifugation (10 min, 10 000 × *g*) between washes.

3.1. 1:3 1-amino-Cer:NFA-GalCer

As above except the lipid assembly (4.9 mg) of tubules was stirred with 0.85 mg BODIPY-FL-NSSE.

4. Synthesis

A variety of headgroups were created through covalent modification of the sphingosine molecule. These headgroups include azide, ethers, silylethers, sulfonyl esters, esters and amides. The synthesis of the analogs began with 24:1-Cer, 16:0-Cer, 8:0-Cer, 3-silyl-24:1-Cer, 1-trityl-24:1-Cer or 1-amino-24:1-Cer as shown in Figs. 1 and 2. These materials are commercially available, described within this paper (1-amino-24:1-Cer), or previously published. (Goldstein et al., 1997).

4.1. C-1 modification of 24:1-Cer

The following materials were generated by reacting the 1° alcohol of 24:1-Cer with a variety of reagents. The 1-azido-24:1-Cer (1) material was prepared, albeit in poor yield, using a zinc azide bispyridine complex (Viaud and Rollen, 1990). 1-*O*-triphenylmethyl-24:1-Cer (2), (Goldstein et al., 1997), 1-*O*-triphenylmethyl-16:0-Cer (3), and 1-*O*-triphenylmethyl-8:0-Cer (4) were formed by treating the appropriate ceramide with triphenylmethylchloride. The 1-*O*-dimethoxytriphenylmethyl-24:1-Cer (5) was prepared using dimethoxytriphenylmethylchloride. 1-*O*-allyl-24:1-Cer (6) and 1-*O*-allyl-16:0-Cer (7) were generated in moderate yields when the 1° alcohol of the appropriate ceramide was reacted with sodium hydroxide and allylbromide. The 1-*O*-*p*-toluenesulfonyl-24:1-Cer (8) was formed, albeit in very

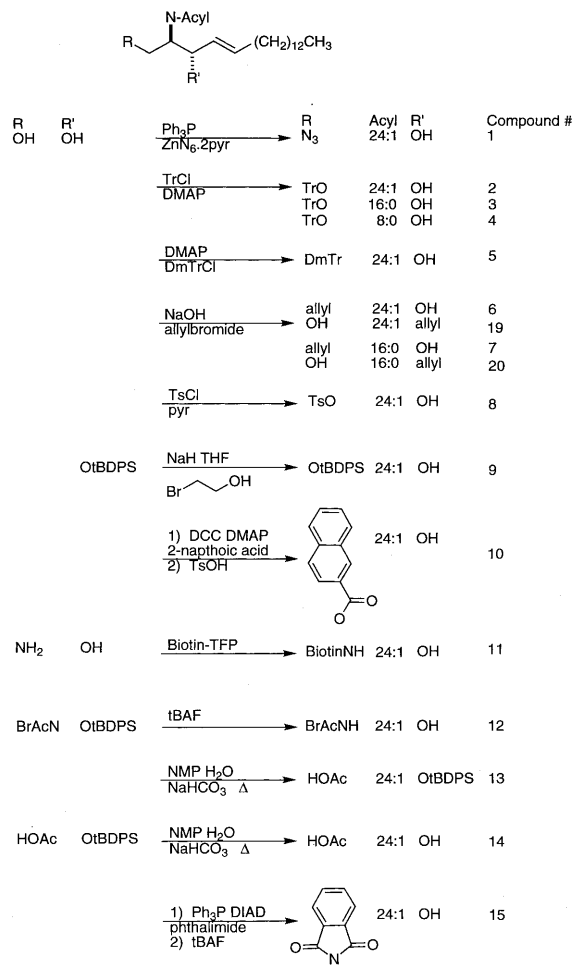

Fig. 1.

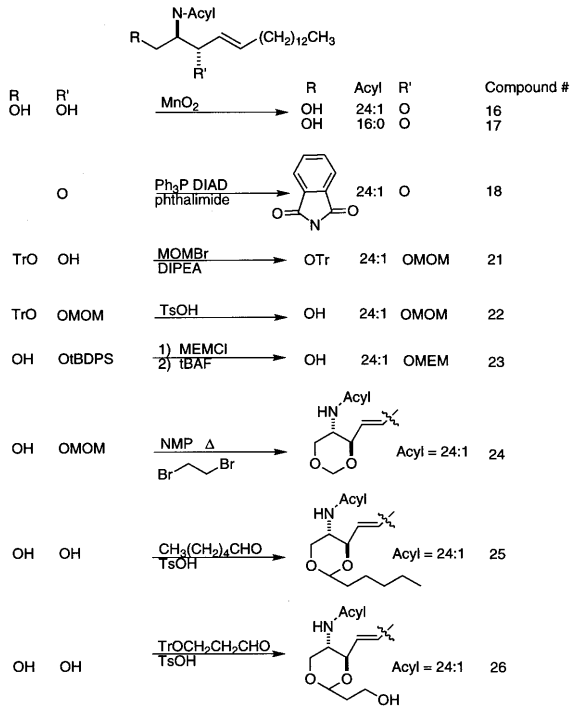

Fig. 2.

poor yield, using the corresponding sulfonyl chloride. A failed Williamson etherification reaction caused migration of the 3-*O*-*t*-butyldiphenylsilyl group to form the 1-*O*-tBDPS-24:1-Cer (9). The 1-*O*-napthoyl-24:1-Cer (10) was prepared using a carbodiimide mediated coupling of napthoic acid with a 3-silyl protected ceramide (Goldstein et al., 1997) followed by acid catalyzed desilylation.

The following synthesized lipids have nitrogen replacing the C-1 oxygen. The 3-silyl protected ceramide (Goldstein et al., 1997) was converted to the 1-phthalimdo-24:1-Cer using Mitsunobu chemistry. The amine was revealed upon treatment with hydrazine and silyl group could be removed using fluoride. 1-Biotin-24:1-Cer (11) material was formed by treatment of 1-amino — 24:1-Cer with biotin-tetrafluorophenyl ester (Wilbur et al., 1997). 1-*N*-bromoacetamido-24:1-Cer (12) was prepared by reaction of the bromoacetic anhydride (Robey and Fields, 1989) with a 1-amino-3-silyl-24:1-Cer followed by fluoride mediated desilylation. The 1-bromoacetamido-3-silyl material was also converted to the 1-hydroxyacetamido-3-silyl-24:1-Cer (13) and the 1-hydroxyacetamido-3-hydroxy-24:1-Cer (14) upon treatment with mild aqueous base in *N*-methylpyrolidine (Hutchins and Taffer, 1983). Treatment of 3-silyl-24:1-Cer with phthalimide under Mitsunobu conditions followed by fluoride desilylation formed 1-phthalimido-24:1-Cer (15).

Ceramides with functionality at the 2° allylic alcohol (C-3) were also generated. The ketone, allyl ether, methoxyethoxymethyl ether, and methoxymethyl ether were prepared. Treatment of 24:1-Cer or 16:0-Cer with manganese oxide generated the respective 3-keto materials (16) and (17). Using a Mitsunobu reaction, 3-keto-24:1-Cer (16) was converted to 1-phthalimido-3-keto-24:1-Cer (18). Treatment of 24:1-Cer or 16:0-Cer with allylbromide and sodium hydroxide also formed the 3-*O*-allyl-24:1-Cer (19) and 3-*O*-allyl-16:0-Cer (20). 1-*O*-triphenylmethyl-24:1-Cer was converted to the 1-*O*-triphenylmethyl-3-*O*-methoxyethoxy methyl material (21) which was also detritylated under mild acid conditions to provide 3-*O*-methoxyethoxymethyl-24:1-Cer (22). Similarly, 1-*O*-tBDPS-24:1-Cer (9) was converted to the 3-*O*-methoxymethyl-24:1-Cer (23) by treatment with methoxymethyl chloride followed by fluoride mediated desilylation.

Ceramides with bridging functionality at C-1 and C-3 were also prepared. Originally, attempts to *O*-alkylate C-1 in the presence of a 3-*O*-methoxymethyl ether (22) under basic conditions resulted in the formation of the 1,3-formyl acetal (24). This unexpected result led to the further investigations involving cyclic acetals. The 1,3-hexyl acetal (25) was prepared by treating 24:1-Cer with hexanal and a mild acid. Lastly, treatment of 24:1-Cer with 3-*O*-triphenylmethyl-propanal and *p*-toluenesulfonic acid generated the 3′-hydroxy-(1,3)-propyl acetal (26).

5. Results and discussion

In all cases, sphingolipids were precipitated from a 1.0 mM DMF solution by the addition of water (35% by volume) at room temperature. This method has been found to be the most likely one to form CHARMs of those tried to date. The

resultant cloudy suspensions were allowed to stand overnight; an aliquot was then removed for examination by TEM. The observed morphologies are summarized in Table 1 and representative example shown in Fig. 3. In 9 of 26 cases tried, no type of recognizable CHARM formed; these precipitates are denoted as 'amorphous'. When CHARMs did form, their morphologies were either ribbons or cylindrical structures. In many cases, the ribbons tended to twist at irregular intervals or fold over onto themselves. In most cases, there existed up to a 2-fold variation in the measured diameter. Since CHARMs tend to form a tangled mesh (Archibald and Mann, 1994; Kulkarni et al., 1995), determining the average length of the supramolecular assemblies was not usually feasible.

A sphingolipid's CHARM morphology may be attributed to specific patterns of inter- and intramolecular H-bonding of the headgroups; therefore, chemical modifications to this region may be expected to affect CHARM topology (Pascher, 1976; Pascher and Sundell, 1977; Pascher et al., 1992; Hamilton et al., 1993; Nandi and Bagchi, 1996; Selinger et al., 1996; Moore and Rerek, 1997). Furthermore, a sphingolipid's hydrocarbon packing energy stabilization may also play a factor in CHARM formation. Since we have no data that bears on sphingolipid intermolecular interactions, it is difficult to determine the exact reason why lipids may or may not form CHARMs; however, some trends appear to be present. In general, sphingolipids lacking a H-bond donor/acceptor attached to C-1 (excluding the C-1 oxygen if present) did not form CHARMs. The 1-azido-24:1-Cer (1), 1-Tr-8:0-Cer (4), 1-DmTr-24:1-Cer (5), and 1-tBDPS-24:1-Cer (9) formed amorphous aggregates. Some of these results are surprising in light of the fact that similar compounds do make supramolecular assemblies. For example, 1-Tr-

Table 1

Compound #	Headgroup descriptor	Morphology	Dimensions
1	1-Azido-24:1	Amorphous	–
2	1-TrO-24:1	CHARM	1.3 μm
3	1-TrO-16:0	Sheet	–
4	1-TrO-8:0	Amorphous	–
5	1-DmTrO-24:1	Amorphous	–
6	1-*O*-allyl-24:1	Amorphous	–
7	1-*O*-allyl-16:0	Ribbon	0.1 μm
8	1-TsO-24:1	Ribbon	–
9	1-*O*-tBDPSi-24:1	Amorphous	–
10	1-Napthoyl-24:1	Hollow tube	0.3 μm
11	1-Biotinamido-24:	Ribbon	0.2 μm
12	1-Bromoacetamido-24:1	CHARM	0.8 μm
13	1-Hydroxyacetamido-3-*O*-tBDPSi-24:1	Amorphous	–
14	1-Hydroxyacetamdo-24:1	CHARM	–
15	1-Phathalimido-24:1	Ribbon	0.2 μm
16	3-Keto-24:1	Ribbon	1.3 μm
17	3-Keto-16:0	Ribbon	50 nm
18	1-Phthalimido-3-keto-24:1	Amorphous	–
19	3-*O*-allyl-24:1	Ribbon	1.7 μm
20	3-*O*-allyl-16:0	Amorphous	–
21	1-TrO-3-OMOM-24:1	CHARM	0.3 μm
22	3-OMOM-24:1	CHARM	0.8 μm
23	3-OMEM-24:1	Amorphous	–
24	1,3-*O*-formylacetal-24:1	Ribbon	85 nm
25	1,3-*O*-hexylacetal-24:1	CHARM	
26	1,3-*O*-(3′-hydroxy)-propylacetal-24:1	Ribbon	10×200 nm

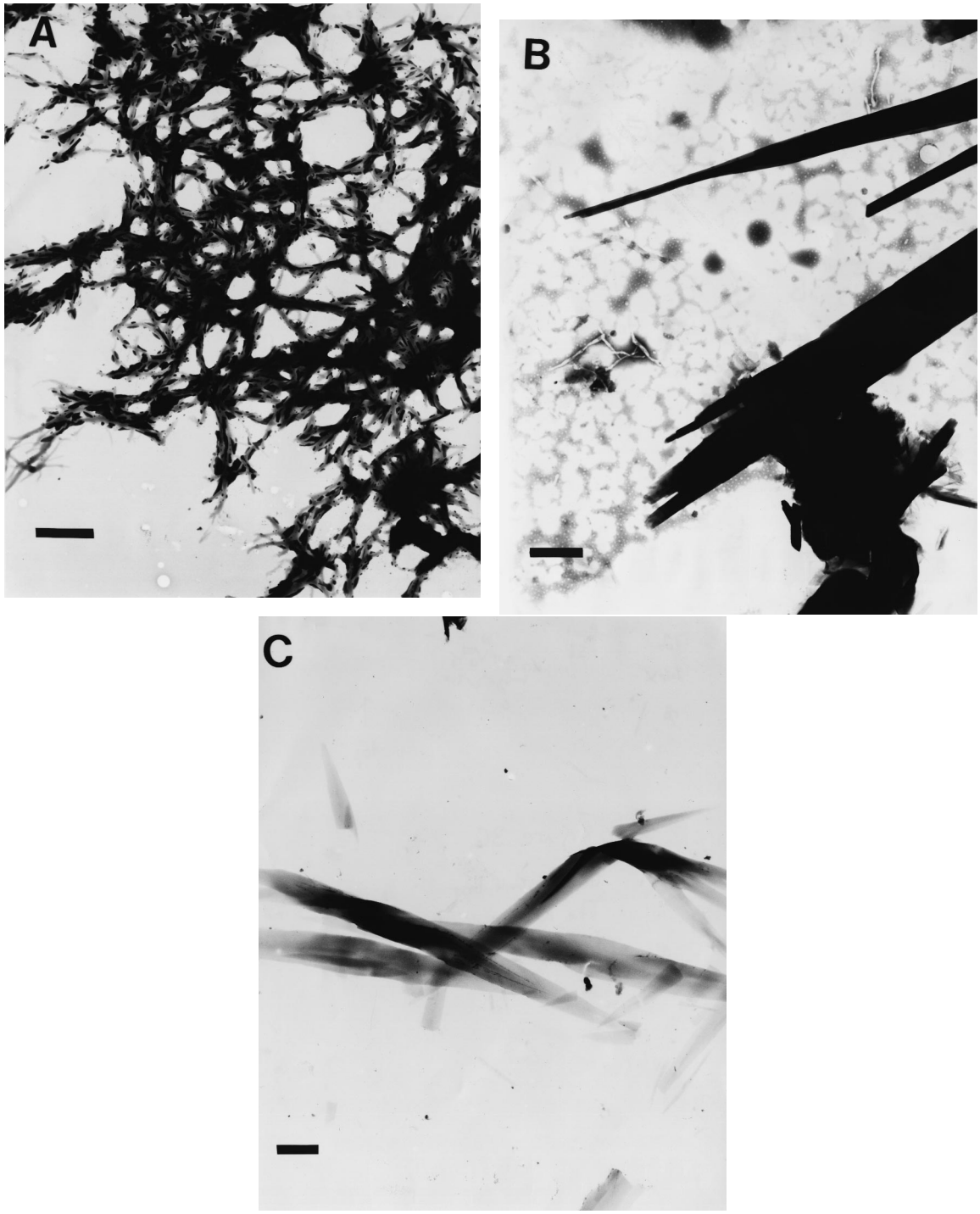

Fig. 3. TEM of (A) bromoacetamido-24:1-Cer (12). Scale bar is 10 μm; (B) 1-biotinamido-24:1-Cer (11). Scale bar is 2 μm; (C) 3-keto-24:1-Cer (16). Scale bar is 10 μm.

24:1-Cer (2) and 1-Tr-16:0-Cer (3) formed CHARMs. On initial examination, one would expect the structurally similar shorter acyl trityl compound (4) and the dimethoxytrityl (5) to aggregate into well defined morphologies. In the first case, it appears that the bulky hydrophobic trityl headgroup may disrupt the tight headgroup packing required for CHARM formation. However, longer alkyl chain sphingolipids (2) and (3) have stronger hydrocarbon interactions that may counteract the disruption by the trityl group. In the dimethoxytrityl case, either the added methoxy groups could exceed the cross-sectional diameter of a sphingolipid, thus preventing favorable headgroup–headgroup packing interactions (large headgroup with small alkyl chains tends to favor H_{II}), or the increased electron density of the methoxyphenyl group may perturb potential energy stabilizing dipole–dipole interactions (π-stacking) between headgroups. When a phenyl group is replaced with a *t*-butyl group, as in the case of 1-tBDPS-24:1-Cer (9), microstructures did not form, perhaps because of the absence of possible π-stacking in the headgroups, supporting the above premise.

A majority of the modified headgroups are similar in size but differ electronically. For example, phthalimide (15), tosyl (8), and napthoyl (10) substituents are ester linked, relatively nonpolar, have significant π-clouds, and formed CHARMs. On the contrary, biotin (11), bromoacetamide (12) and hydroxyacetamide (14) lipids are amide linked, relatively polar but lack significant π-clouds, they also formed CHARMs. Perhaps additional H-bond donor/acceptors in the amide linked lipids (as compared with potential π-stacking in the aromatic headgroups) are sufficient to permit CHARM formation, or the hydrocarbon interactions give sufficient stabilization.

In almost all cases, two different *N*-acyl groups, 16:0 (palmitoyl) and 24:1 (nervonoyl), were employed. Palmitoyl, being full saturated, can exist in an all-*trans* configuration, the lowest hydrocarbon energy state. Nervonoyl contains a *cis* double bond so the hydrocarbon tail has a 'kink' that perturbs the hydrocarbon packing. The hydrocarbon packing differences may be responsible for differences in the morphologies of 1-*O*-allyl-24:1-Cer (6), 1-*O*-allyl-16:0-Cer (7), 3-*O*-allyl-24:1-Cer (19), and 3-*O*-allyl-16:0-Cer (20).

The allyl group is hydrophobic but relatively small, so it may try to 'dive' into the hydrophobic region causing significant rearrangement of the headgroup. Since 1-*O*-allyl-24:1-Cer (6) already has a perturbed hydrocarbon region, further rearrangement of the H-bonding network of the headgroup and hydrocarbon packing may not favor CHARM formation, resulting in amorphous aggregates. On the other hand, 1-*O*-allyl-16:0-Cer (7), existing in an all *trans* configuration, may be able to better reorganize its hydrocarbon chains to accommodate the small headgroup, thus allowing CHARM formation.

When the allyl group was moved to the C-3 position, the 3-*O*-allyl-24:1-Cer (19) formed CHARMs, whereas 3-*O*-allyl-16:0-Cer (20) did not form CHARMs. In these cases, if the allyl group dives into the hydrophobic region the headgroup does not have to significantly rearrange. For the longer chain amphiphile (19) the greater hydrocarbon length allows for greater flexibility in accommodating packing perturbances than the shorter hydrocarbon chains of amphiphile (20).

Other chemical modifications at C-3 of the hydrophilic regions can be tolerated. For example, the C-3 keto compounds (16) and (171 formed ribbonlike CHARMs. The size of the attached hydrophilic group is important. The smaller methoxymethyl material (22) did form CHARMs whereas, the methoxyethoxymethyl group (23), which contains one additional methylene, did not form microstructures.

It also appears that dual modification within the hydrophilic region can be tolerated. 1-Trityl-3-MOM-24:1-Cer (21) formed CHARMs. Acetals, which include the C-1 and C-3 oxygens in a six membered ring can form microstructures. The acetals can be hydrophilic such as 1,3-formylacetal (24) or 1,3-prophylhydroxyacetal (26) or hydrophobic such as 1,3-hexylacetal (25).

6. Conjugation to preformed CHARMs

Since not all headgroup-modified sphingolipids can independently form CHARMs, it is advanta-

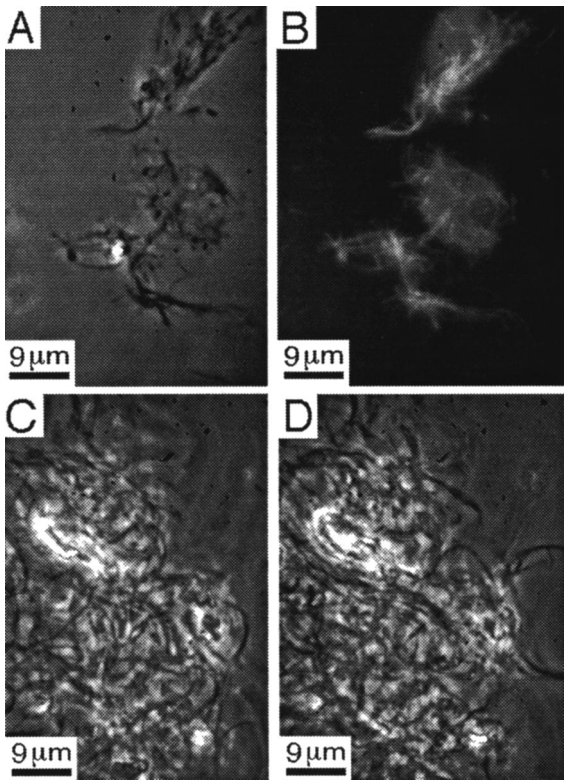

Fig. 4. Optical micrographs of (A) BODIPY treated NFA-GalCer:1-amino-24:1-Cer CHARMs viewed under white light; (B) BODIPY treated NFA-GalCer:1-amino-24:1-Cer CHARMs viewed under fluorescent light; (C) BODIPY treated NFA-GalCer CHARMs viewed under white light; (D) BODIPY treated NFA-GalCer CHARMs viewed under fluorescent light.

geous to have a method with which to covalently attach the drug to preformed CHARMs. CHARMs composed of the nonhyxdroxy fatty acid portion of galactocerebroside (NFA-GalCer) or the 1:3 mixture of 1-amino-24:1-Cer and NFA-GalCer were used as models for such a template. CHARMs were treated with an activated fluorescent carboxylic acid (BODIPY); only the amine should react with the fluorophore for conjugation. After incubation, the supernatants were removed, and the nanostructures washed with brine to remove any noncovalently associated BODIPY. As shown in Fig. 4, optical micrographs of the 'ropes' of NFA-GalCer assemblies did not have any significant fluorescence associated with the microstructures whereas the mixed lipid system did fluoresce. In fact, CHARMs with amine present at only 0.5 mol% were successfully conjugated (not shown) and all the amine was reagent accessible when treated with 5-fold excess of activated fluorophore. When the fluorescent-CHARMs are dissolved in solvent and re-precipitated, the BODIPY-amine-sphingolipid is excluded from the CHARMs.

7. Conclusions

Sphingolipid-based CHARMs tolerate a wide variety of chemical moieties within the headgroup region. Headgroups may include hydrophobic and hydrophilic moieties and may be cyclic or acyclic in nature. Based on the few compounds that failed to form CHARMs used in this study, it appears that as long as the H-bonding network of the hydrophilic headgroup is not significantly perturbed, regular microstructures of some sort can form. A sphingolipid with an amine-containing headgroup may be chemically modified after CHARM formation. These results suggest that a wide range of therapeutic moieties could be incorporated into CHARMs for potential use as single or multiple (co)drug delivery systems. The insolubility of the long chain sphingolipids may immobilize the CHARM at the desired site of action, thus serving as a local drug depot and increasing the residence time of hydrophilic drugs. This is under exploration in our group at this time.

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