Effects of sphingosine 2*N*- and 3*O*-methylation on palmitoyl ceramide properties in bilayer membranes

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1 MATERIAL AND METHODS

1.1 Material

1-Palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) was from Avanti Polar Lipids and *N*-palmitoyl-D-*erythro*-ceramide (PCer) from Larodan Fine Chemicals (Malmö, Sweden). Cholesterol (CHL), methyl-β-cyclodextrin (mβCD), sphingomyelinase (*S. aureus*) and Dulbecco's phosphate buffered saline (PBS) were from Sigma/Aldrich (St. Louis, MO, USA). *Trans*-parinaric acid (tPA) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Cholesta-5,7,9(11)-trien-3-beta-ol (CTL) was synthesized and purified as described previously (1). 1-Palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) was synthesized from 7-doxyl-stearic acid (TCI Europe) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) in an esterification reaction as described previously (2). tPA, CTL and 7SLPC were stored dry under argon at –87°C until dissolved in argon-purged methanol, ethanol or hexane:isopropanol (3:2 by vol), respectively, and used within a week. All lipid stock solutions were prepared in hexane:isopropanol (3:2 by vol), stored at –20°C and warmed to ambient temperature before use. The water used for sample preparation was purified by reverse osmosis followed by passage through a Millipore UF Plus water-purification system (Millipore, Billerica, MA) to yield a product with final resistivity of 18.2 MΩcm. All other organic and inorganic chemicals were of highest purity available, and solvents of spectroscopic grade.

1.2 DSC

Multilamellar vesicles for DSC were prepared by mixing the desired lipids (PSM or PSM/XCer, 1:1 by mol) and evaporating the solvent under a stream of nitrogen. Residual solvent was removed under vacuum for 1 h. The samples were then hydrated with MQ-water (final lipid concentration 1 mM) at 95°C for 30 min, followed by sonication at 95°C (Bransonic 2510 bath sonicator, Branson Ultrasonics, Danbury, CT) with several rounds of intermittent vigorous vortex mixing until opalescent and homogeneous preparations were obtained. Samples were cooled down to room temperature before transfer to the DSC. Four consecutive heating and cooling scans were recorded between 20°C and 95°C at a temperature gradient of 1°C/min using a high-sensitivity Microcal VPDSC-instrument (Northampton, MA, USA). Data were analyzed with Origin-software (OriginLab, Northampton, MA, USA).

1.3 Fluorescence quenching experiments

Samples (50 μM total lipid) for fluorescence quenching experiments were prepared by mixing the lipids (POPC/PSM/CHL, 60:30:10 or 75/15/10, or POPC/PSM/XCer/CHL 60:15:15:10 by mol), fluorescent probes (CTL or tPA) and the quencher (7SLPC) and drying

under a stream of nitrogen. Residual solvent was removed in vacuum for 30 min. The dry lipid films were stored under argon at –20°C until hydrated one at a time for 30 min with pre-heated, argon-purged MQ-water at 65°C. Multilamellar vesicles were then formed at 65°C by sonicating the CTL-containing samples with a probe sonifier (W-450, Branson Ultrasonics, Danbury, CT, USA) for 2 min, and the tPA-containing samples with a Branson bath sonicator for 5 min. For each quenching curve two samples were prepared, the quenched F-sample and the unquenched F_0-sample. In addition to the desired lipids the F-samples contained 1 mol% of either tPA or CTL, and 30 mol% of 7SLPC which replaced an equal amount of POPC. The F_0-samples contained no 7SLPC but only 1 mol% of tPA or CTL in addition to the lipids under study. Quenching data were collected with a QuantaMaster-1-spectrofluorometer (Photon Technology International, Lawrenceville, NJ, USA) by measuring the fluorescence signal of tPA (Ex 305 nm, Em 405 nm) or CTL (324nm/390 nm) under constant stirring while heating the samples at a rate of 5°C/min.

1.3.1 Analysis of the trans-parinaric acid quenching data

A 2D-smoothing of the tPA-quenching data was performed with Sigma Plot-software. The negative exponential algorithm and nearest neighbour bandwidth method were used. The temperature limits were set from 10°C to 60°C for all samples and 200 intervals were collected with a sampling proportion of 0.1. A polynomial degree of two was used in all cases. From the smoothed quenching curves, the onset temperature for domain melting was determined as the corresponding temperature at the point where the slope of the F/F_0-ratio started to decline. The offset for domain melting was determined as the corresponding temperature at the point where the F/F_0-ratio started to level off or reached the lowest point. For some samples the F/F_0 started to increase again after reaching the lowest point due to increased background noise at low fluorescence signal intensities (originating from the quenched sample). This was not considered as a sample-induced relevant change in quenching susceptibility of the fluorophores. The T_m was determined as the corresponding temperature at the midway between the onset and offset values of F/F_0.

1.4 tPA fluorescence life-time measurements

Fluorescence life-times of tPA were measured in multilamellar vesicles (0.1 mM final lipid concentration containing 0.5 mol% of tPA) of varying lipid composition (POPC/XCer, 85:15 or POPC/PSM/XCer/CHL, 60:15:15:10 by mol). The vesicles were otherwise formed as described in the DSC method, but after hydration the samples were vortexed briefly, saturated with argon and sonicated for 10 min at 65°C (Branson bath sonicator). Before fluorescence measurements the samples were kept in the dark at RT over night. The fluorescence decays of tPA were recorded at 23°C with a FluoTime 200-spectrometer with a PicoHarp 300E time-correlated single photon counting module (PicoQuant GmbH, Berlin, Germany). tPA was excited with a 298 nm led laser source and the emission collected at 430 nm. The samples were kept under constant stirring during the measurements. Data were acquired and analyzed with the FluoFit Pro-software obtained from PicoQuant.

1.5 Sterol partitioning assay

Sterol partitioning between large unilamellar vesicles and mβCD was performed as described previously (3, 4). Shortly, lipids were mixed (POPC/PSM/CHL, 60:30:10 or 75/15/10, or POPC/PSM/XCer/CHL, 60:15:15:10 by mol, including 2 mol% CTL), and dried under a stream of nitrogen. The lipid films were then hydrated for 30 min at 65°C and vortexed briefly to

form multilamellar vesicles. The multilamellar vesicles were then extruded through a membrane with 200 nm pores to yield unilamellar vesicles, which were used for the partitioning assay to obtain the molar fraction partitioning coefficient (K_x).

2 Synthesis of 2*N*(methyl)-palmitoyl-D-*erythro*-ceramide (NMeCer)

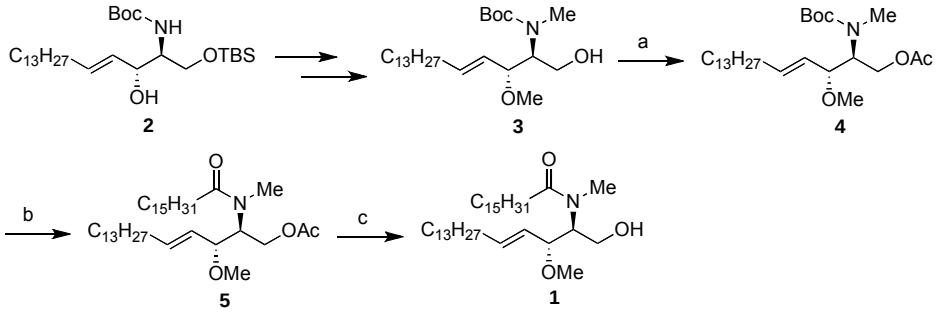
NMeCer was produced by hydrolysis of 2*N*(methyl)-palmitoyl-D-*erythro*-sphingomyelin (NMeSM, prepared as described previously (5)) with *S. aureus* sphingomyelinase (SMase) in a two-phase system (6) as follows: NMeSM was dissolved in organic solvent (ethylacetate/hexane, 1:1 by vol, containing 1.8 % v/v ethanol), the mixture was heated to 41°C and mixed with the water phase (6 % PBS, pH adjusted to 7.4, containing 8 U of SMase). After incubation for 2 hours at 41°C the organic solvent phase was collected, dried under a stream of nitrogen and purified by reverse-phase HPLC (Supelco Discovery C18-column, dimensions 250 × 21.2 mm, 5 µm particle size, Bellefonte, PA) eluted with methanol/hexane (95:5, by vol, with additional 5 % of water) as the mobile phase (flow 9 ml/min) and UV-detection at 203 nm. NMeCer was identified by mass spectrometry on a Bruker Daltonics Ion Trap-ESI-MS (Bremen, Germany) and the purity of the product was verified by Merck/Hitachi LaChrome-analytical HPLC (Supelco Discovery C18-column, dimensions 125 × 4.6 mm, 5 µm particle size, Bellefonte, PA).

3 Synthesis of 3*O*(methyl)-*N*-palmitoyl-ceramide (OMeCer)

OMeCer was produced from 3-hydroxymethyl-*N*-palmitoyl-D-*erythro*-sphingomyelin (OMeSM, prepared as described previously (5)) by acid hydrolysis (7) followed by reacylation (8). First, OMeSM was hydrolyzed in water/methanol (1:9, by vol, with 1 M HCl) at 100°C over night, resulting in breakdown of the OMeSM into phosphorylcholine, 3-hydroxymethyl-sphingosine and fatty acid methyl ester. The hydrolyzed products were then extracted with hexane and the remaining water phase became basic with NaOH (pH ~12) followed by extraction with diethylether. The ether phase, containing the 3-hydroxymethyl-sphingosine was then dried under a stream of nitrogen and re-coupled to palmitic anhydride in the acylation reaction at 45°C over night. The synthesized product was purified by reverse-phase HPLC with methanol as the mobile phase (as above). Purity of the OMeCer was verified by analytical HPLC and identity by ESI-MS.

4 Synthesis of 2*N*(methyl)-3*O*(methyl)-palmitoyl-D-*erythro*-ceramide (NMeOMeCer)

NMeOMeCer **1** was synthesized from *N*-*t*-butoxycarbonyl-*O*-*t*-butyldimethylsilyl-sphingosine **2** through the known compound **3**. The efficient synthetic method of **2** from L-serine was already established. The transformation of **3** into **1** was accomplished by the following sequences: after the primary hydroxyl group of **3** was protected by acetylation to give **4**, whose Boc group was transformed into an acyl group to afford **5**. Finally, hydrolysis of the acetyl group afforded NMeOMe ceramide **1**.



Scheme. a) Ac_2O, DMAP, pyr, rt, 81%; b) TFA, CH_2Cl_2, 0°C, then sat. $NaHCO_3$ aq., $C_{15}H_{31}COCl$, $CHCl_3$, rt, 57% for 2 steps; c) 2N NaOH aq., MeOH, rt, 87%.

4.1 Experimental details for synthesis of NMeOMeCer

Synthesis of **4**: to a solution of **3** (0.291 g, 0.681 mmol) in pyridine (3 ml) was added acetic anhydride (0.13 ml, 1.36 mmol) and 4-dimethylaminopyridine (0.017 g, 0.14 mmol) at room temperature. After the reaction mixture was stirred at the same temperature for 30 min, a solution of $CuSO_4 \cdot 5H_2O$ was added and the resulting mixture was extracted with ethyl acetate. The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo to give the crude products. Column chromatography on silica gel (from 2 % to 5 % ethyl acetate in hexane) gave **4** (0.258 g, 81 %) as a colourless oil. $[\alpha]^{24.0}_D$ –24.0 (c 0.435, $CHCl_3$); IR (ZnSe, cm^{-1}); 2928, 2856, 1738, 1686, 1456, 1090 ; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 5.67 (dt, *J* = 15.3, 6.6, Hz, 1H), 5.28 (dd, *J* = 15.1, 8.5 Hz, 1/2H), 5.22 (dd, *J* = 15.6, 8.7 Hz, 1/2H), 4.44 - 4.07 (m, 3H), 3.69 - 3.61 (m, 1/2H), 3.60 - 3.51 (m, 1/2H), 3.21 (s, 3/2H), 3.20 (s, 3/2H), 2.74 (s, 3/2H), 2.72 (s, 3/2H), 2.04 (dt, *J* = 6.9, 6.9 Hz, 2H), 1.45 (s, 9/2H), 1.44 (s, 9/2H), 1.39 - 1.25 (m, 22H), 0.88 (t, *J* = 6.6 Hz, 3H) ; ^{13}C NMR (100 MHz, $CDCl_3$) δ: 155.8 (1/2C), 155.5 (1/2C), 136.8 (1/2C), 136.4 (1/2C), 127.0 (1/2C), 126.7 (1/2C), 81.8 (1/2C), 81.6 (1/2C), 79.4 (1/2C), 79.1 (1/2C), 61.7, 55.9, 32.2, 31.8, 29.6, 29.5, 29.3, 29.2, 29.1, 29.0, 28.3, 22.5, 20.6, 13.9 ; ESI-HRMS m/z calculated for $C_{27}H_{51}N_1O_5Na$ $(M+Na)^+$ 492.3665, found 492.3685.

Synthesis of **5:** to a solution of **4** (0.161 g, 0.343 mmol) in dichloromethane (3 ml) was added trifluoroacetic acid (0.6 ml) at 0°C. After the reaction mixture was stirred at the same temperature for 1 h, it was concentrated in vacuo to give the crude products. To a solution of the obtained crude products in chloroform (3 ml) was added a saturated aqueous $NaHCO_3$ solution under ice-cooling until the pH of the solution became basic. Palmitoyl chloride (0.11 ml, 0.36 mmol) was added to the resulting mixture at room temperature. After the reaction mixture was stirred at the same temperature for 15 min, a saturated aqueous $NaHCO_3$ solution was added and the resulting mixture was extracted with ethyl acetate. The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo to give the crude products. Column chromatography on silica gel (from 2 % to 17 % ethyl acetate in hexane) gave **5** (0.118 g, 57 %) as a colourless solid. $[\alpha]^{24.0}_D$ –17.0 (c 0.470, $CHCl_3$); IR (ZnSe, cm^{-1}); 2928, 2854, 1738, 1635, 1456, 1092 ; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 5.71 (dt, *J* = 15.1, 6.6, Hz, 1/2H), 5.67 (dt, *J* = 15.3, 6.6 Hz, 1/2H), 5.24 (dd, *J* = 15.1, 8.5 Hz, 1/2H), 5.21 (dd, *J* = 15.3, 8.2 Hz, 1/2H), 4.43 (dd, *J* = 11.4, 11.0 Hz, 1/2H), 4.38 - 4.26 (m, 2H), 4.04 (td, *J* = 12.6, 4.4 Hz, 1/2H), 3.70 - 3.60 (m, 1/2H), 3.57 - 3.52 (m, 1/2H), 3.23 (s, 3/2H), 3.20 (s, 3/2H), 2.87 (s, 3/2H), 2.73 (s, 3/2H), 2.31 (td, *J* = 7.6, 2.7 Hz, 1H), 2.24 (td, *J* = 7.3, 1.6 Hz, 1H), 2.07 - 1.99 (m, 2H), 2.02 (s, 3H), 1.65 – 1.61 (m, 2H), 1.38 - 1.20 (m, 46H), 0.88 (t, *J* = 6.6 Hz, 6H) ; ^{13}C NMR (100 MHz, $CDCl_3$) δ: 174.0 (1/2C), 173.9 (1/2C), 170.9 (1/2C), 170.6 (1/2C), 127.0 (1/2C), 126.0 (1/2C),

81.9, 61.6, 59.2, 56.3, 56.1, 34.1, 33.4, 32.3, 32.0, 29.8, 29.6, 29.4, 29.2, 28.0, 25.4, 25.1, 22.7, 20.9, 20.8, 14.2 ; ESI-HRMS m/z calculated for $C_{38}H_{73}N_1O_4Na$ $(M+Na)^+$ 630.5437, found 630.5426.

Synthesis of **1:** to a solution of **5** (0.075 g, 0.123 mmol) in methanol (2 ml) was added an aqueous 2N NaOH solution (0.24 ml) at room temperature. After the reaction mixture was stirred at the same temperature for 10 min, it was acidified with an aqueous 0.1N HCl solution and the resulting mixture was extracted with chloroform. The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo to give the crude products. Column chromatography on silica gel (from 10 % to 33 % ethyl acetate in hexane) gave **1** (0.061 g, 87 %) as a colourless solid. $[\alpha]^{24.0}_D$ –21.6 (c 0.305, $CHCl_3$); IR (ZnSe, cm^{-1}); 3437, 3305, 2926, 2854, 1716, 1456, 1441, 1082 ; ^1H NMR (400 MHz, $CDCl_3$), δ: 5.74 - 5.63 (m, 1H), 5.27 (dd, *J* = 15.3, 8.7 Hz, 1/2H), 5.22 (dd, *J* = 15.6, 8.2 Hz, 1/2H), 4.11 - 4.02 (m, 1/2H), 3.98 - 3.82 (m, 3H), 3.78 - 3.63 (m, 1/2H), 3.26 (s, 3/2H), 3.25 (s, 3/2H), 2.95 (s, 3/2H), 2.76 (s, 3/2H), 2.41 - 2.18 (m, 2H), 2.08 - 1.99 (m, 2H), 1.64 - 1.54 (m, 2H), 1.34 - 1.21 (m, 46H), 0.88 (t, *J* = 6.6 Hz, 6H) ; ^{13}C NMR (100 MHz, $CDCl_3$) δ: 174.8 (1/2C), 174.3 (1/2C), 137.7 (1/2C), 136.9 (1/2C), 127.4 (1/2C), 126.5 (1/2C), 83.1, 81.9, 62.2, 61.9, 56.3, 34.4, 33.8, 32.4, 32.1, 29.8, 29.7, 29.6, 29.5, 29.4, 29.1, 28.4, 25.5, 25.1, 14.2 ; ESI-HRMS m/z calculated for $C_{36}H_{71}N_1O_3Na$ $(M+Na)^+$ 588.5332, found 588.5309.

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