Is the Linkage Region of Sphingolipids Responsible for Lipid Raft Formation?

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Supporting Information

General Methods. Unless stated, otherwise, all reagents were obtained from commercial sources, and used without further purification. Phosphorous trichloride was refluxed and distilled before use. Triethylamine was distilled from CaH_2 and stored over KOH. Pivaloyl chloride was distilled prior to use. THF, toluene and pyridine were used in an anhydrous form. All ^{1}H-, ^{31}P, and ^{13}C-NMR spectra were recorded on a Bruker 360 MHz instrument; chemical shifts are reported in ppm and are referenced to residual solvent. Phosphoric acid was used as external reference for ^{31}P NMR. D-*erythro*-sphinganine (Avanti Polar Lipids) was used as received.

***N*-Myristoyl-D-*erythro*-Sphinganine (5)** D-*erythro*-sphinganine (1.02 g, 3.38 mmol) was dissolved in 30 mL of THF, followed by addition of 0.95 mL of Et_3N (6.76mmol, 2eq), and a solution of 1.22 g of *N*-succinimidyl tetradecanoate (3.72 mmol, 1.1eq). After stirring for 15 h, the precipitate was removed by filtration. The organic layer was then concentrated under reduced pressure, and the crude product purified by recrystallization from 5~10mL of EtOH to give 1.50 g of colorless solid (86%), having R_f 0.47 ($CHCl_3$/MeOH, 10/1, v/v); ^{1}H NMR (360 MHz, $CDCl_3$) δ: 6.33 (d, *J*=7.2 Hz, 1 H), 4.00 (dd, *J*=3.4, 10.4 Hz, 1 H), 3.81 (m, 1 H), 3.76 (m, 2 H), 2.21 (t, *J*=7.6 Hz, 2 H), 1.71 (m, 2 H), 1.62 (m, 2 H), 1.51 (m, 2 H), 1.23 (m, 46 H), 0.85 (t, *J*=6.7 Hz, 3 H); ^{13}C NMR (90 MHz, $CDCl_3$) δ: 173.7, 74.0, 62.3, 53.7, 45.7, 36.7, 34.3, 31.8, 29.5, 29.2, 25.9, 25.7, 22.6, 14.0; IR ν_{max} (KBr) 3278, 2918, 2850, 1727, 1640, 1552, 1465, 1211, 1072cm^{-1}; HRMS for $C_{32}H_{66}NO_3$ Calcd: 512.5043. Found: 512.5035.

***N*-Myristoyl-1-O-benzoyl-3-triisopropylsilyl-D-erythro-sphinganine (6)**. Sphinganine **5** (316 mg, 0.62 mmol) was dissolved in 1.0 mL of pyridine, and 15.0 mg (0.31 mmol) of DMAP and 72.0 μL (0.62 mmol) of benzoyl chloride were added, successively. The reaction mixture was stirred for 15 h at room temperature. Removal of solvent under reduced pressure and purification of the residue by column chromatography (SiO_2, 30 g, $CHCl_3$/CH_3OH, 50/1 and 20/1, v/v) afforded 60.5 mg (19% recovery) of unreacted **5** and 252.3 mg (66%) of a monobenzoylated product as a colorless solid, having R_f 0.76 ($CHCl_3$/CH_3OH, 10/1, v/v); ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.99 (d, *J*=5.0 Hz, 2 H), 7.56 (t, *J*=7.4 Hz, 1 H), 7.43 (t, *J*=7.5 Hz, 2 H), 5.97 (d, *J*=8.4 Hz, 1 H), 4.62 (dd, *J*=6.7, 11.7 Hz, 1 H), 4.40 (dd, *J*=3.7, 11.7 Hz, 1 H), 4.28 (m, 1 H), 3.67 (m, 1 H), 2.17 (t, *J*=7.4 Hz, 2 H), 1.57 (m, 2 H), 1.51 (m, 2 H), 1.23 (m, 44 H), 0.85 (t, *J*=6.7 Hz, 3 H); ^{13}C NMR (90 MHz, $CDCl_3$) δ 173.5, 149.1, 136.4, 133.2, 129.6, 128.4, 72.6, 63.4, 53.1, 36.8, 34.0, 31.8, 29.6, 29.3, 26.0, 25.7, 22.6, 14.0; IR ν_{max} (KBr) 3284, 2922, 2851, 1730, 1640, 1552, 1465, 1278, 1120, 1073, 705 cm^{-1}.

To a solution of 40.8 mg (0.066 mmol) of the monobenzoylated sphinganine and 38.5 μL (0.33 mmol, 5 equiv) of 2,6-lutidine in 2.0 mL of THF was added 53.5 μL of TIPSOTf (0.20 mmol), dropwise, at 0°C. The reaction mixture was then stirred for 15 min at 0°C and warmed to room temperature for 2.5 h. Removal of solvent under reduced pressure, followed by column chromatography (SiO_2, 25g, hexane/EtOAc, 10/1, v/v) afforded 39.5 mg (77%) of **6** having R_f 0.56 (hexane/EtOAc, 4/1, v/v); ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.99 (d, *J*=7.2 Hz, 2 H), 7.52 (t, *J*=7.7 Hz, 1 H), 7.39 (t, *J*=7.2 Hz, 2 H), 5.88 (d, *J*=5.9 Hz, 1 H), 4.4 (m, 3 H), 4.01 (t, *J*=7.2 Hz, 1 H), 2.10 (m, 2 H), 1.62 (m, 2 H), 1.55 (m, 2 H), 1.23 (m, 42 H), 1.04 (m, 21 H), 0.85 (t, *J*=6.8 Hz, 6 H); ^{13}C NMR (90 MHz, $CDCl_3$) δ 172.5, 166.8, 132.9, 129.7, 128.3, 74.4, 62.6, 50.9, 37.0, 34.9, 31.9, 29.6, 29.3, 25.6, 25.3, 22.6, 18.2, 14.0, 12.8; IR ν_{max} (KBr) 3272, 2920, 2852, 1726, 1639, 1555, 1467, 1268, 1113, 883, 709cm^{-1}. HRMS for $C_{48}H_{90}NO_4Si$ Calcd: 772.6639. Found: 772.6663

***N*-Myristoyl-*N'*-*tert*-butyloxycarbonyl-3-O-TIPS-D-*erythro*-sphinganine (7)**. To a solution of 168.3 mg (022 mmol) of **6** in 2 mL of CH_2Cl_2 was added a solution of NaOMe in MeOH [2.0 mg of Na (0.08 mmol) in 0.5 mL of anhydrous MeOH]. After stirring for 14 h at room temperature, the mixture was diluted with Et_2O and passed through 15 g of silica gel by washing with Et_2O several times. The solvent was removed under reduced pressure, and the residue purified by column chromatography (SiO_2, 15 g, hexane/EtOAc, 10/1 to 4/1, v/v) to give 112.5 mg (77%) of the corresponding primary alcohol as colorless oil having Rf 0.21 (hexane/EtOAc, 4/1, v/v); ^{1}H NMR (360 MHz, $CDCl_3$) δ 6.33 (d, *J*=7.2 Hz, 1 H), 3.8-4.1 (m, 3 H), 3.58 (dd, *J*=2.9, 10.8 Hz, 1 H)), 2.18 (t, *J*=7.5 Hz, 2 H), 1.61 (m, 4 H), 1.23 (m, 45 H), 1.06 (m, 21 H), 0.86 (t, *J*=6.8 Hz, 6 H); ^{13}C NMR (90 MHz, $CDCl_3$) δ 173.1, 62.2, 52.2, 36.8, 34.9, 31.9, 29.6, 29.3, 25.5, 25.3, 22.6, 18.1, 14.0, 12.6; IR νmax (KBr) 3295, 2952, 2850, 1643, 1557, 1467, 1120, 1061cm-1.

To a suspension of 92.5 mg (1.96 mmol) of imidazole in 2.0 ml of toluene was added 62.0 μL of PCl_3 (0.41 mmol) dropwise at 0°C. After stirring the mixture for 15 min at 0°C, 152.0 μL of Et_3N (1.09 mmol) was added dropwise over 5 min. After an additional 15 min of stirring, a solution of 91.0 mg (0.136 mmol) of the debenzoylated form of **6** in 2.0 mL of toluene was added, dropwise, over 5 min at 0°C, using 1 mL of toluene as a rinse. After 2 h of stirring at 0°C, 0.5 mL of H_2O was added to quench the product mixture. The mixture was then diluted with EtOAc, washed with 30 mL of H_2O, 30 mL of saturated NaCl, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was dried azeotropically with toluene several times, and then dissolved in 10 mL of pyridine. To this mixture was added 66.0 mg (0.408 mmol) of *N*-(*tert*-butoxycarbonyl)ethanolamine, followed by 84 μL (0.68 mmol) of pivaloyl chloride. The resulting solution then stirred at room temperature for 1.5 h. To the reaction mixture was then added 34.5 mg (0.136 mmol) of I_2 and 1.5 mL of H_2O. After stirring for 1.5 h at room temperature, a saturated solution of $Na_2S_2O_3$ was added to quench the reaction. The product mixture was diluted with CH_2Cl_2, washed with 40 mL of saturated $Na_2S_2O_3$, 40 mL of saturated NaCl, dried over Na_2SO_4, and concentrated under reduced pressure. Purification by column chromatography (SiO_2, 15 g, $CHCl_3$/CH_3OH, 20/1 and $CHCl_3$/CH_3OH/H_2O, 65/25/1, v/v/v) yielded 61.7 mg (51%) of **7** as a colorless oil having R_f 0.58 ($CHCl_3$/CH_3OH/H_2O, 13/6/1, v/v/v); ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.10 (brs, 1 H), 4.02 (brs, 1 H), 3.94 (brs, 1 H), 3.81 (brs, 3 H), 3.20 (brs, 2 H), 2.15 (m, 2 H), 1.52 (brs, 4 H), 1.39 (s, 9 H), 1.22 (m, 44 H), 1.03 (m, 21 H), 0.85 (t, *J*=7 Hz, 6 H); ^{13}C NMR (90 MHz, $CDCl_3$) δ 174.0, 156.3, 78.6, 73.5, 64.9, 63.8, 53.0, 41.3, 36.8, 34.4, 31.9, 29.7, 29.3, 28.4, 25.7, 24.8, 22.6, 18.2, 14.0, 12.8; ^{31}P NMR (145 MHz, $CDCl_3$) δ 0.08; IR ν_{max} (KBr) 3432, 2926, 2857, 1691, 1643, 1523, 1463, 1245, 1104, 1065cm^{-1}. HRMS for $C_{48}H_{98}N_2O_8PSiNa_2$ Calcd: 935.6626. Found: 935.6582.

***N*-[3-(2-Pyridyldithio)propionyl]-D-erythro-sphinganine (8)**. To a solution of **7** (376.4 mg, 0.42 mmol) in 10.0 mL of CH_2Cl_2 and 2 mL of CH_3CN, which was added 50 drops (ca. 2.5 mL) of HF-Pyr complex. The mixture was stirred for 1 h at room temperature, and passed through 20 g of silica using $CHCl_3$/CH_3OH (20/1, v/v) and $CHCl_3$/CH_3OH/H_2O, (13/6/1,

v/v/v). The organic layer was concentrated under reduced pressure, and the residue was then dissolved in 10 mL of CH_2Cl_2, followed by addition of 1 mL of trifluoroacetic acid. The reaction mixture was stirred for an additional 1 h at room temperature, and the solvent then removed under reduced pressure. The crude product was dried, azeotropically, with MeOH and toluene several times prior to the next reaction. The crude oil was then dissolved in 10 mL of $CHCl_3$, and 0.75 mL of Et_3N (5.4 mmol) added, followed by addition of a solution made from 167.7 mg (0.46 mmol) of *N*-[O-1,2,3-benzotriazin-4(3H)one-yl]-3-(2-pyridyldithio)propionate [BPDP] plus 3 mL of $CHCl_3$, using 2 mL of $CHCl_3$ as a rinse. The reaction mixture was stirred for 15 h, diluted with $CHCl_3$, washed with 50 mL of saturated aqueous $NaHCO_3$, 50 mL of saturated NaCl, dried over Na_2SO_4, and concentrated under reduced pressure. Subsequent purification by column chromatography (SiO_2, 25 g, $CHCl_3/CH_3OH$, 20/1 to $CHCl_3/CH_3OH/H_2O$, 13/6/1, v/v/v), followed by preparative TLC (Merck, SiO_2, 2 mm, 20×20 cm, $CHCl_3/CH_3OH/H_2O$, 65/25/1, v/v/v) afforded 198.3 mg (56%, 3 steps) of **8** as a colorless oil having R_f 0.57 ($CHCl_3/CH_3OH/H_2O$, 13/6/1, v/v/v); ^{1}H NMR (360 MHz, $CDCl_3/CD_3OD$, 1/1) δ 8.36 (m, 1 H), 7.81 (m, 1 H), 7.74 (m, 1 H), 7.15 (t, *J*=5.8 Hz, 1 H), 4.21 (m, 1 H), 3.88 (m, 2 H), 3.80 (m, 2 H), 3.59 (m, 1 H), 3.40 (m, 2 H), 3.03 (t, *J*=7.1 Hz, 2 H), 2.60 (t, *J*=7.1 Hz, 2 H), 2.18 (t, 6.6 Hz, 2 H), 1.57 (m, 2 H), 1.49 (m, 2 H), 1.22 (m, 46 H), 0.84, (t, *J*= 7.5 Hz, 6 H); ^{13}C NMR (90 MHz, $CDCl_3/CD_3OD$, 1/1) δ 174.5, 172.5, 160.6, 149.7, 138.5, 121.7, 120.6, 70.1, 65.2, 64.6, 54.8, 40.8, 36.9, 35.6, 34.9, 34.0, 32.5, 30.2, 30.0, 29.9, 26.6, 26.4, 23.2, 14.3. ^{31}P NMR (145 MHz, $CDCl_3/CD_3OD$, 1/1) δ: 4.99; IR ν_{max} (KBr) 3289, 2919, 2849, 1647, 1554, 1468, 1419, 1232, 1006 cm^{-1}. HRMS for $C_{42}H_{77}N_3O_7PS_2Na_2$ Calcd: 876.4736. Found: 876.4698.

Sphingolipid Homodimer 1. To a solution of 89.5 mg (0.72 mmol) of dithiothreitol (DTT) in 5.0 mL of $CHCl_3$ was added a solution of 30 mg (0.036 mmol) of **8** in 2.0 mL of $CHCl_3$, dropwise at 0°C under a nitrogen atmosphere. The reaction mixture was stirred for 30 min at 0°C and the solvent was then removed under reduced pressure. The resulting thiol monomer was purified by column chromatography (SiO_2, 5 g, $CHCl_3/CH_3OH$, 20/1 to $CHCl_3/CH_3OH/H_2O$, 13/6/1, v/v/v), and then by preparative TLC (Merck, SiO_2, 0.5 mm, 20×20 cm, $CHCl_3/CH_3OH/H_2O$, 13/6/1, v/v/v). The resulting thiol monomoer was dissolved in 3 mL of $CHCl_3$, and a solution of 33 mg (0.04 mmol) of **8** in 2.5 mL of $CHCl_3$ was added dropwise at room temperature; 1.0 mL of $CHCl_3$ was used as a rinse. The mxiture was stirred for 18 h, concentrated under reduced pressure and the residue purified by preparative TLC (Merck, SiO_2, 0.5 mm, 20 × 20 cm, $CHCl_3/CH_3OH/H_2O$, 13/6/1, v/v/v) to give 22.8 mg (44%) of **1** as a colorless oil having R_f 0.48 ($CHCl_3/CH_3OH/H_2O$, 13/6/1, v/v/v); ^{1}H NMR (360 MHz, $CDCl_3/CD_3OD$, 1/1, v/v) δ 4.06 (m, 2 H), 3.80 (m, 8 H), 3.50 (m, 2 H), 3.36 (m, 4 H), 2.90 (m, 4 H), 2.55 (m, 4 H), 2.13 (m, 4 H), 1.52 (m, 4 H), 1.45 (m, 4 H), 1.18 (m, 92 H), 0.80 (t, *J*=6.6 Hz, 12 H). ^{13}C NMR (90 MHz, $CDCl_3/CD_3OD$ (1/1), δ 175.1, 173.0, 70.2, 65.2, 64.6, 54.6, 40.7, 36.9, 35.9, 34.5, 34.0, 32.4, 30.2, 29.8, 26.5, 26.3, 23.1, 14.3; ^{31}P NMR (145 MHz, $CDCl_3/CD_3OD$, 1/1, v/v) δ 3.16; IR ν_{max} (KBr) 3420, 3300, 2923, 2854, 1646, 1555, 1466, 1242, 1103, 1068 cm^{-1}. HRMS for $C_{74}H_{147}N_4O_{14}P_2S_2Na_2$ Calcd: 1487.9620. Found: 1487.9674.

Heterodimer 4. Using procedures similar to those used for the synthesis of **1**, the corresponding heterodimer (**4**) was prepared by reacting **8** with 1 equiv of the thiol monomer of **2** in 47 % isolated yield, having R_f 0.50 ($CHCl_3$/MeOH/H_2O, 13/6/1, v/v/v); ^{1}H-NMR (360MHz, $CDCl_3/CD_3OD$, 1/1) δ: 5.19 (m, 1 H), 4.40 (d, *J*=10.0 Hz, 1 H), 4.14 (m, 2 H), 3.8-4.1 (m, 8 H), 3.59 (m, 1 H), 3.40 (m, 4 H), 2.92 (t, *J*=6.8 Hz, 4 H), 2.59 (t, *J*=7.0 Hz, 4 H), 2.28 (dd, *J*=7.3, 12.4 Hz, 4 H), 2.18 (t, *J*=7.4 Hz, 2 H), 1.53 (m, 8 H), 1.22 (m, 102 H), 0.85 (t, *J*=6.9 Hz, 12 H); ^{13}C NMR (90 MHz, $CDCl_3/CD_3OD$, 1/1) δ: 175.3, 174.5, 174.2, 173.1, 70.9, 70.3, 65.5, 64.9, 64.4, 63.1, 54.7, 40.7, 36.9, 36.1, 34.6, 34.1, 32.5, 30.2, 29.9, 29.7, 26.6, 26.3, 25.4, 23.2, 14.3. ^{31}P NMR (145 MHz, $CDCl_3/CD_3OD$, 1/1) δ: 3.44, 2.37. IR ν_{max} (KBr) 3292, 2920, 2853, 1741, 1653, 1558, 1468, 1238, 1099, 1066cm^{-1}. HRMS for $C_{81}H_{157}N_3O_{16}P_2S_2Na_3^+$ Calcd: 1623.0168. Found: 1623.0204