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Transfection Efficacies of α-Tocopherylated Cationic Geminis with Hydroxyethyl bearing Headgroups in High Serum Conditions

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Synthetic Procedure and Characterization Details.

Ethyl 2-tocopheryloxy acetate (1). In an ice cooled condition a solution of $(\pm) \alpha$ -tocopherol (10 g, 23.25 mmol) was dissolved in dry DMF (20 mL) and sodium hydride (0.84 g, 35 mmol) was added with stirring. After 15 min of stirring ethyl bromoacetate (5.83 g, 35 mmol) was added dropwise. After overnight stirring at room temperature the solvent was removed in vacuum with heating. The compound was dissolved in ethyl acetate (500 mL), washed with water (2 X 100 mL) and brine (2 X 100 mL). The separated organic solvent was dried over anhydrous sodium sulfate and evaporated to get a crude residue. Compound was purified by column chromatography with eluting pet ether and ethyl acetate (v/v 100:4) solvent mixture. Isolated yield was 7.2 g, 60%. FT-IR (Neat, cm⁻¹) 3053, 2925, 2865, 2360, 1757, 1458, 1414, 1373, 1262, 1204, 11.58, 1204, 1093, 869, 734; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.87 (m, 12H, -CH-CH₃, phytyl chain), 1.07-1.83 (m, 26H), 1.31-1.35 (t, 3H), 2.08 (s, 3H, -CH₃), 2.15 (s, 3H, -CH₃), 2.19 $(s, 3H, -CH_3)$, 2.58 (t, 2H, J = 6.8 Hz), 4.27- 4.32 (m, 2H), 4.3 (s, 2H); ¹³C NMR (100 MHz, $CDCl_3$) δ 11.76, 11.84, 12.70, 14.2, 19.57, 19.59, 19.64, 19.66, 19.72, 20.59, 20.99, 22.60, 22.70, 23.83, 24.41, 24.78, 24.79, 27.96, 31.15, 31.19, 32.63, 32.65, 32.75, 32.77, 37.26, 37.34, 37.36, 37.38, 37.43, 39.34, 40.02, 40.03, 61.02, 70.00, 74.86, 117.60, 122.98, 125.61, 127.53, 147.77, 148.18, 169.40. HRMS (ESI) m/z calcd for $[C_{33}H_{56}O_4 + Na]^+$: 539.4076; found: 539.4076.

2-Tocopheryloxy ethanol (2).¹ Ethyl 2-tocopheryloxyacetate (7.2 g, 13.95 mmol) was dissolved in dry THF (30 mL) and cooled in 0 °C. Then lithium aluminum hydride (0.558 g, 13.95 mmol) was added to it and stirred at room temperature for 6h. The reaction was quenched by adding ethyl acetate and ice water. Mixture was diluted with ethyl acetate (200 mL). Then organic solvent was separated and then washed with1 N HCl (2 X 30 mL), water (2 X 30 mL) and brine (2 X 30 mL). Ethyl acetate layer was then passed through anhydrous sodium sulfate and

evaporated in vacuum to get a crude product. Compound was purified by column chromatography with eluting pet ether and ethyl acetate (v/v 80:20). Isolated yield was 5.95 g, 90%. FT-IR (Neat, cm⁻¹) 3425, 3053, 2925, 2865, 2117, 2008, 1981, 1602, 1457, 1414, 1376, 1262, 1159, 1088, 1037, 894, 734, 705; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.87 (*m*, 12H, -CH-CH₃, phytyl chain), 1.07-1.83 (*m*, 26H), 2.07 (*s*, 3H, -CH₃), 2.13 (*s*, 3H, -CH₃), 2.17 (*s*, 3H, -CH₃), 2.58 (*t*, 2H, *J* = 6.8 Hz), 3.49 (*s*, 1H), 3.78 (*t*, 2H, *J* = 6.4), 3.93- 3.94 (*m*, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 11.76, 11.84, 12.70, 19.57, 19.60, 19.66, 19.73, 20.62, 21.02, 22.61, 22.70, 23.85, 24.42, 24.78, 27.96, 29.68, 31.16, 31.21, 32.65, 32.67, 32.75, 32.77, 37.26, 37.36, 37.38, 37.43, 37.55, 39.34, 40.03, 40.07, 62.42, 73.62, 74.83, 117.61, 122.97, 125.69, 127.66, 147.56, 147.95. HRMS (ESI) m/z calcd for [C₃₁H₅₄O₃ + Na]⁺: 497.3971; found: 497.3971.

2-Tocopheryloxyethyl tosylate (3). To a solution of compound 2 (5.95 g, 12.55 mmol) in dichlomethane (30 mL), pyridine (1.3 mL) and TsCl (3.59 g, 18.8 mmol) were added with stirring at 0 °C. Then the resulting mixture was stirred at room temperature for 6h. The organic solvent was then removed in vacuum to leave a residue which was further dissolved in ethyl acetate (500 mL). Organic layer was washed with 1N HCl (2 X 100 mL), saturated NaHCO₃ (2 X 100 mL), and water (2 X 100 mL). The organic solvent from the separated layer was dried over anhydrous sodium sulfate and evaporated to get the crude product. Compound 3 was purified by column chromatography while eluting with pet ether and ethyl acetate (v/v 100:4). Isolated yield was 6.7 g, 85%. FT-IR (Neat, cm⁻¹) 3054, 2962, 2866, 1597, 1457, 1417, 1262, 1175, 1091, 1017, 921, 814, 734, 704, 663; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.87 (*m*, 12H, -CH-C<u>H</u>₃, phytyl chain), 1.07-1.83 (*m*, 26H), 2.05 (*s*, 9H, -CH₃), 2.08 (*s*, 3H, -CH₃), 2.44 (*s*, 3H), 2.54 (*t*, 2H, *J* = 6.8 Hz), 3.85 (*t*, 2H, *J* = 4.8 Hz), 4.33 (*t*, 2H, *J* = 4.4 Hz), 7.33-7.35 (*d*, 2H, *J* = 8 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 11.72, 11.77, 12.63, 19.55, 19.65,

19.72, 20.57, 21.00, 21.62, 22.60, 22.69, 23.81, 24.40, 24.77, 27.97, 31.13, 31.18, 32.64, 32.66, 32.76, 37.25, 37.30, 37.42, 37.54, 39.33, 39.99, 40.03, 69.09, 69.93, 74.84, 117.59, 122.95, 125.71, 127.61, 127.96, 129.81, 132.99, 144.79, 147.37, 148.04. HRMS (ESI) m/z calcd for $[C_{38}H_{60}O_5S + Na]^+$: 651.4059; found: 651.4059.

2-(N-(2-Tocophervloxvethyl)-N-methylamino)ethanol (4).² To a solution of compound 3 (6.7 g, 10.67 mmol) in dry acetonitrile (15 mL) and ethanol (3 mL) N-methyl ethanolamine was mixed and the mixture was refluxed for 12h. After the completion of the reaction, solvent was evaporated to afford a residue which was diluted with chloroform (500 mL). The chloroform layer was washed repeatedly with water (2 X 100 mL), brine (2 X 100 mL) and finally filtered through anhydrous sodium sulphate for drying. The filtrate was then evaporated in vacuum to get a crude residue. The solid was purified by column chromatography upon eluting with chloroform and methanol (v/v 100:4) solvent mixture. The isolated yield was 3.97 g, 70%. FT-IR (Neat, cm⁻¹) 3420, 3053, 2925, 2865, 2361, 2171, 2008, 1981, 1602, 1475, 1414, 1376, 1262, 1159, 1088, 1037, 894, 734, 705; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.87 (m, 12H, -CH-CH₃, phytyl chain), 1.07-1.83 (m, 26H), 2.07 (s, 9H, -CH₃), 2.13 (s, 3H, -CH₃), 2.17 (s, 3H, -CH₃), 2.56 (s, 3H), 2.56 (t, 2H, J = 6.8 Hz), 2.68 (t, 2H, 5.2 Hz), 2.87 (t, 2H, J = 5.6 Hz), 3.64 (t, 2H, J = 5.2 Hz), 3.75 (t, 2H, J = 5.2 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 11.75, 11.88, 12.75, 19.59, 19.65, 19.71, 20.62, 20.99, 22.59, 22.69, 23.83, 24.40, 24.78, 27.94, 31.02, 31.25, 32.64, 32.66, 32.74, 37.25, 37.35, 37.42, 37.54, 39.33, 39.97, 40.01, 42.38, 57.09, 58.56, 59.22, 70.07, 74.76, 117.53, 122.87, 125.67, 127.65, 147.79, 148.16. HRMS (ESI) m/z calcd for $[C_{34}H_{61}NO_3 + H]^+$: 532.4731; found: 532.4731.

General Synthesis of Gemini Lipids. Compound 4 (0.2 g, 2.5 equiv) was dissolved in acetonitrile in a screw top pressure tube and α,ω -alkane diyl dibromide (1 equiv) was added to

this. The mixture was stirred at 80 °C for 2 weeks. Solvent was then evaporated to get crude product. The crude residue was purified by column chromatography over neutral alumina upon eluting with chloroform and methanol mixture (v/v 100:6). The isolated yields ranged from 25 to 30% of the hygroscopic lipids.

TH4S: FT-IR (Neat, cm⁻¹) 3345, 2924, 2859, 1596, 1456, 1375, 1265, 1162, 1019, 916;; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.87 (*m*, 24H), 1.07-1.83 (*m*, 52H), 2.03 (*s*, 6H), 2.09 (*s*, 6H), 2.12 (*s*, 6H), 2.20 (*m*, 4H), 2.52 (*s*, 4H), 3.50 (*s*, 6H), 3.88 (*s*, 4H), 4.05 (*s*, 12H), 4.2 (*s*, 4H); ¹³C NMR (100 MHz, CDCl₃) δ 11.79, 12.57, 13.37, 19.90, 20.62, 21.01, 21.73, 22.60, 22.70, 23.57, 24.41, 24.77, 27.93, 29.66, 31.01, 32.69, 32.74, 37.24, 37.35, 37.41, 39.32, 40.09, 40.17, 50.49, 55.73, 64.16, 66.03, 74.98, 117.84, 123.30, 125.12, 126.90, 147.38, 148.48; HRMS (ESI) m/z calcd for $[C_{72}H_{130}N_2O_6]^{2+}/2$: 559.495; found: 532.4981; Anal. Calcd for $C_{72}H_{130}N_2O_6Br_2.2H_2O$: C 65.73, H 10.27, N 2.13. Found: C 65.79, H 10.30, N 2.08.

TH5S: FT-IR (Neat, cm⁻¹) 3347, 2924, 2860, 1595, 1478, 1441, 1266, 1117, 1020, 912; ¹H NMR (400 MHz, CDCl₃) δ 0.82-0.86 (*m*, 24H), 1.06-1.79 (*m*, 54H), 2.04 (*s*, 6H), 2.09(*s*, 6H), 2.12 (*s*, 6H), 2.16 (*m*, 4H), 2.53 (*m*, 4H), 3.5 (*s*, 6H), 3.91-4.17 (*m*, 20H); ¹³C NMR (100 MHz, CDCl₃) δ 11.79, 12.52, 13.34, 19.54, 19.59, 19.65, 19.72, 20.64, 21.01, 21.85, 22.60, 22.70, 23.62, 24.42, 24.77, 27.94, 31.11, 32.69, 32.75, 37.25, 37.36, 37.42, 37.57, 39.33, 40.12, 50.48, 55.82, 63.92, 64.12, 66.10, 75.00, 117.89, 123.36, 125.10, 126.89, 147.40, 148.51.; HRMS (ESI) m/z calcd for $[C_{73}H_{132}N_2O_6]^{2+}/2$: 566.5037; found: 566.4904; Anal. Calcd for $C_{73}H_{132}N_2O_6Br_2.3H_2O$: C 65.06, H 10.32, N 2.08. Found: C 64.99, H 9.87, N 2.42.

TH6S: FT-IR (Neat, cm⁻¹) 3343, 2924, 1594, 1458, 1373, 1265, 1162, 1092, 1019, 916; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.86 (*m*, 24H), 1.07-1.8 (*m*, 56H), 2.05 (*s*, 6H), 2.10 (*s*, 6H), 2.13 (*s*, 6H), 2.13-2.06 (*m*, 4H), 2.53 (*m*, 4H), 3.52 (*s*, 6H), 3.84-4.19 (*m*, 20H) ; ¹³C NMR (100 MHz,

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CDCl₃) δ 11.78, 12.49, 13.32, 19.52, 19.58, 19.64, 19.71, 20.62, 21.00, 22.59, 22.68, 23.61, 24.40, 24.75, 24.86, 27.92, 29.65, 31.09, 31.88, 32.68, 32.73, 37.23, 37.34, 37.40, 37.54, 39.31, 40.00, 40.09, 50.44, 55.84, 62.78, 64.08, 64.28, 66.12, 74.98, 117.85, 123.33, 125.10, 126.89, 147.41, 148.47. HRMS (ESI) m/z calcd for $[C_{74}H_{134}N_2O_6]^{2+}/2$: 573.5115; found: 573.5128; Anal. Calcd for $C_{74}H_{134}N_2O_6Br_2.0.15H_2O$: C 67.83, H 10.33, N 2.14. Found: C 67.80, H 10.02, N 2.44. **TH8S**: FT-IR (Neat, cm⁻¹) 3324, 2925, 2862, 1596, 1459, 1374, 1266, 1161, 1090, 1018, 918; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.87 (*m*, 24H), 1.07-1.83 (*m*, 60H), 1.95 (*m*, 4H), 2.06 (*s*, 6H), 2.10 (*s*, 6H), 2.14 (*s*, 6H), 2.55 (*m*, 4H), 3.48 (*s*, 6H), 3.84 - 4.19 (*m*, 20H); ¹³C NMR (100 MHz, CDCl₃) δ 11.66, 12.36, 13.18, 19.41, 19.45, 19.47, 19.52, 19.54, 19.60, 20.52, 20.89, 22.00, 22.48, 22.57, 23.47, 24.29, 24.64, 25.41, 27.54, 27.81, 31.03, 31.09, 32.57, 32.61, 32.62, 37.12, 37.25, 37.29, 37.36, 37.45, 39.21, 39.97, 40.06, 50.21, 55.67, 62.39, 64.34, 66.04, 74.84, 117.71, 123.16, 125.03, 126.82, 147.38, 148.33. HRMS (ESI) m/z calcd for $[C_{76}H_{138}N_2O_6]^{2+}/2$: 587.5272; found: 587.5229; Anal. Calcd for $C_{76}H_{138}N_2O_6Br_2.3.4H_2O$: C 65.34, H 10.45, N 2.01. Found: C 65.41, H 10.42, N 1.98.

TH12S: FT-IR (Neat, cm⁻¹) 3346, 2923, 2858, 1596, 1459, 1456, 1267, 1160, 1090, 1019, 915; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.87 (*m*, 24H), 1.06-1.83 (*m*, 68H), 1.88 (*s*, 4H), 2.06 (*s*, 6H), 2.10 (*s*, 6H), 2.13 (*s*, 6H), 2.55 (*m*, 4H), 3.49 (*s*, 6H), 3.76-4.17 (*m*, 20H); ¹³C NMR (100 MHz, CDCl₃) δ 11.77, 12.37, 13.20, 19.62, 19.69, 20.61, 20.98, 22.48, 22.57, 22.66, 23.63, 24.38, 24.74, 25.96, 27.91, 28.48, 28.53, 28.60, 32.66, 32.71, 37.21, 37.32, 37.38, 37.51, 39.30, 40.06, 50.13, 55.80, 62.38, 64.39, 64.58, 66.12, 74.98, 117.85, 123.34, 125.03, 126.83, 147.38, 148.48. HRMS (ESI) m/z calcd for [C₈₀H₁₄₆N₂O₆]²⁺/2: 615.5585; found: 615.5706; Anal. Calcd for C₈₀H₁₄₆N₂O₆Br₂.3H₂O: C 65.45, H 10.60, N 1.94. Found: C 65.50, H 10.57, N 1.98.

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2-Tocopheryloxyethyl bromide (5). To a solution of 2-tocopheryloxyethyl tosylate (0.628g, 1 mmol) in dry acetone, LiBr (0.174g, 2 mmol) was added. The resulting solution was refluxed for 12h. Upon completion of the reaction, solvent was evaporated and diluted with 200 mL chloroform. Chloroform layer was washed with water (2 X 30 mL), brine (2 X 20mL) and dried over anhydrous sodium sulfate. Solvent was evaporated in vacuum to get crude product which was further purified with column chromatography by eluting with pet ether and ethyl acetate as solvent (v/v 100:10). Isolated yield was 0.4 g, 75%. FT-IR (Neat, cm⁻¹); 2926, 2862, 1459, 1413, 1374, 1335, 1252, 1161, 1161, 1094, 1009, 918; ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.87 (m, 12H, -CH-CH₃, phytyl chain), 1.07-1.83 (m, 26H), 2.07 (s, 3H, -CH₃), 2.14 (s, 3H, -CH₃), 2.18 $(s, 3H, -CH_3), 2.56 (t, 2H, J = 6.8 Hz), 3.63 (t, 2H, J = 6.4 Hz), 3.96 (t, 2H, J = 6.4 Hz);$ ¹³C NMR $(100 \text{ MHz}, \text{CDCl}_3) \delta 11.77, 11.98, 12.85, 19.58, 19.61, 19.67, 19.74, 20.63, 21.02, 21.62, 22.60,$ 22.71, 23.85, 24.42, 24.79, 27.96, 29.64, 29.69, 30.21, 31.20, 31.24, 31.61, 32.65, 32.67, 32.76, 32.78, 37.28, 37.28, 37.32, 37.40, 37.45, 37.54, 40.01, 40.04, 72.29, 74.82, 117.58, 122.98, 125.70, 127.65, 147.47, 148.05. HRMS (ESI) m/z calcd for $[C_{31}H_{53}BrO_2 + Na]^+$: 559.3127, 561.3106; found: 559.3129, 561.3109.

Monomeric Lipid (THM).²

To a solution of 2-tocopheryloxyethyl bromide (0.27 g, 0.5 mmol) in acetonitrile N, N-dimethyl ethanolamine (0.1 g, 1.1 mmol) was added and the mixture was refluxed for 4 days. Then, solvent was evaporated in vacuum and the solid residue was purified by column chromatography upon elution with chloroform and methanol (v/v 100:6) solvent mixture. Isolated yield was 0.24 g, 76%. FT-IR (Neat, cm⁻¹) 3355, 2952, 2858, 1598, 1460, 1374, 1265, 1162, 1089, 1015, 920; ¹H NMR (400 MHz, CDCl₃) δ 0.82-0.86 (*m*, 12H, -CH-C<u>H</u>₃, phytyl chain), 1.06-1.82 (*m*, 26H), 2.05 (*s*, 3H, -CH₃), 2.11 (*s*, 3H, -CH₃), 2.14 (*s*, 3H,-CH₃), 2.54 (*t*, 2H, *J* = 6.4 Hz), 3.59 (*s*, 6H),

4.00 (*s*, 2H), 4.09 (*s*, 2H), 4.13 (*s*, 2H), 4.14 (*s*, 2H), 4.25 (*s*, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 11.79, 12.58, 13.38, 19.52, 19.55, 19.63, 19.70, 20.63, 20.99, 22.58, 22.67, 23.66, 24.39, 24.74, 24.75, 27.92, 29.64, 31.01, 32.67, 37.23, 37.29, 37.33, 37.39, 37.53, 39.31, 39.94, 40.03, 53.53, 53.92, 65.63, 66.15, 67.32, 75.02, 117.91, 123.39, 125.03, 126.82, 147.37, 148.55. HRMS (ESI) m/z calcd for [C₃₅H₆₄NO₃]⁺: 546.4881, found: 546.4834; Anal. Calcd for C₃₅H₆₄NO₃Br.2H₂O: C 63.42, H 10.43, N 2.11. Found: C 63.36, H 10.45, N 2.08.

¹H NMR Spectra of gemini lipids.







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Fig. S1 TE of tocopheryl liposomes in HepG2 cell line in terms of luciferase protein expression in 10% FBS condition at N/P ratios of 0.5-2.



Fig. S2A-E Optimization of DOPE content of co-liposomes in HepG2 cell line for luciferase protein expression in 10% FBS. Data for DOPE molar ratios 0.5:1 to 2:1 at N/P ratios 0.5, 1, 1.5 and 2 are shown.



Fig. S3 Variation of sizes of lipoplexes at various N/P ratios (0.5-2) of optimized co-liposomes (DOPE-THnS = 2:1).



Fig. S4A-E Agarose gel electrophoresis for lipoplexes of DOPE-THnS (2:1) co-liposomes where n = 4, 5, 6, 8 and 12 respectively. The co-liposomes were complexed with pDNA (0.2 µg) and run on 1% agarose gel for ~30 min at 80V.



Fig. S5A Small angle x-ray diffraction pattern of lipoplexes with DOPE-gemini lipid coliposomes (DOPE:THnS = 2:1) at N/P ratio 1.5. **B.** X-ray diffraction pattern of co-liposomes (DOPE:THnS = 2:1). **C.** Comparison of bilayer width of DOPE co-liposomes and their lipoplexes (N/P = 1.5). lipoplex is mentioned as 'lpx'.



Fig. S6 Cellular internalization of lipoplexes (at N/P ratio of highest transfection) with fluorescein labeled pGL3 in A549 cell lines. The co-liposomal formulations of DOPE-TH8S (2:1), DOPE-THM (2:1), DOPE-T8T (4:1) and L2K were used here. The confocal microscopy images were taken after 6h of treatment with the lipoplexes.



Fig. S7 Cellular internalization of lipoplexes (at N/P ratio of highest transfection) with fluorescein labeled pGL3 in A549 cells in presence of different endocytic pathway inhibitors (CPZ, GNT, amiloride and m- β -CD). The co-liposomal formulations of DOPE-TH8S (2:1), DOPE-T8T (4:1) and DOPE-THM (2:1) were used in this experiment.

References

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