Supporting Information

Synthesis of Diacylglycerol Analogs Bearing Photoaffinity Tags Using a Modular Attachment Strategy.
Methods Summary

Photoaffinity probe synthesis. Probes were synthesized in ten steps from commercially available diethyl-L-tartrate using standard organic synthesis techniques, including chromatographic purification and characterization by NMR and mass spectrometry.

DGK purification. *Homo sapiens* DGK-θ was expressed recombinantly in HEK293T cells and purified by affinity chromatography, as described.

Incorporation of photoaffinity probes into liposomes. Photoaffinity probes were resuspended in chloroform, combined with other lipids, and dried to a film under nitrogen gas. The lipid film was hydrated with DGK activity assay buffer, sonicated, and extruded through a 100 nm filter to produce liposomes. All steps with photoaffinity probes were protected from sunlight.

Irradiation. Purified DGK was preincubated with liposomes containing photoaffinity probes (or not, for control), then placed into wells of a 96-well plate on ice, and irradiated with a 360 nm lamp, with or without aluminum foil between the sample and the lamp. Ice controls were not under the lamp and were protected from ambient light. The volume of the samples were remeasured after irradiation to account for evaporation.

DGK activity assay. Samples were incubated with substrate-containing liposomes (as well as probe-containing liposomes if necessary, so at the time of the assay, all samples in the same assay had the same final liposome components) and 32P-γ-ATP. Lipids were extracted and separated by thin layer chromatography (TLC). The location of the 32P-PA on the TLC plate was visualized by autoradiography, and the silica from that location was scraped from the plate and subjected to scintillation counting. Mean ± standard deviation is shown.

Statistical analysis. *p* values were calculated by one-way analysis of variance (Holm-Sidak method, SigmaPlot 12).

Protein LC/MS/MS. Irradiated DGK was separated from other assay components by SDS-PAGE and visualized by silver staining. The band was excised from the gel, destained, and digested in-gel with trypsin. Peptides were removed from the gel and fractionated by reverse-phase HPLC (Magic C18AQ), and eluting peptides were sprayed directly into an LTQ Orbitrap Velos. Peptide sequences were identified from isotopically resolved masses in MS and MS/MS spectra using Mascot (www.matrixscience.com) and Proteome Discoverer (http://portal.thermo-brims.com/).

Lipid MS/MS. Compound 4 was sprayed directly into an AB Sciex 4000 Q Trap in positive ion mode.

Experimental

General. All reagents were purchased from Aldrich or Acros and used as received. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230-400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using a Bruker AC250 spectrometer updated with a TecMag data collection system, a Varian Mercury 300 spectrometer, and
a Bruker Avance 400 spectrometer. Mass spectra were obtained with a JEOL DARTAccuTOF spectrometer with high resolution capabilities. Optical rotation values were obtained using a Perkin-Elmer 241 polarimeter. Compounds 6-9, 14 and 16 were synthesized following previously reported procedures.

(2S,3S)-1-Azido-4-(4-methoxybenzoyloxy)butane-2,3-diol (11). To a stirred solution of compound 9 (2.86 g, 15.3 mmol) in 100 mL N,N′-dimethylformamide (DMF) was added sodium hydride (1.22 g, 30.6 mmol, 60 %), and the solution was allowed to stir at 0 °C for 30 min. Next, para-methoxybenzyl chloride (PMBCl, 4.17 mL, 30.6 mmol) was added to this solution, and the reaction was allowed to stir at rt for 2.5 hrs before it was quenched by adding 3 mL of methanol. The solvent was next removed under reduced pressure. Ethyl acetate (250 mL) was added to dissolve the crude and the solution was extracted from saturated ammonium chloride solution (200 mL), brine (200 mL), and then water (200 mL). The organic layer was dried with magnesium sulfate, filtered and concentrated to yield the crude product 10, which was used in the next reaction without further purification. Crude 10 was redissolved in 10 mL methanol, to which was added 0.40 g of Dowex® 50WX8(100-200 mesh) hydrogen ion-exchange resin. The reaction was allowed to stir at rt overnight and the resin was then filtered off and the filtrate was concentrated to yield crude 11. This was purified by column chromatography with a gradient solvent system of 40-80% ethyl acetate to hexanes to yield compound 11 as colorless oil (1.925 g, 60% over 2 steps). The characterization of the compound matched previously reported values.1 H NMR (300 MHz, CDCl3) δ 7.24 (d, J = 8.5 Hz, 2H), 6.87 (d, J = 8.5 Hz, 2H), 4.50-4.40 (m, 2H), 3.80 (s, 3H), 3.72 (s, 1H), 3.55-3.53 (m, 2H), 3.37-3.33 (m, 2H), 3.17-3.15 (m, 2H), 1.56-1.54 (m, 4H), 1.25-1.23 (m, 52H), 0.87 (t, J = 6 Hz, 6H).

1-(((2S,3S)-4-azido-2,3-bis(hexadecyloxy)butoxy)methyl)-4-methoxybenzene (12). To a cold solution of diol 11 (74 mg, 0.277 mmol) in 10 mL dry N,N′-dimethylformamide was added sodium hydride (66 mg, 1.66 mmol, 60%). The solution was stirred at 0 °C for 30 minutes under nitrogen. Next, 1-bromohexadecane (0.34 mL, 1.11 mmol) and tert-butyl ammonium iodide (TBAI, catalytic) were added to the solution, which was then stirred at rt overnight. The reaction was next quenched by adding 2 mL of methanol. Ethyl acetate (150 mL) was added to the solution and extracted with saturated sodium chloride (100 mL), brine (100 mL) and water (100 mL). The organic layer was dried with magnesium sulfate, filtered and concentrated to yield the crude product, which was purified by column chromatography with a gradient solvent system of 2-20% ethyl acetate to hexanes to yield compound 12 as colorless oil (190 mg, 96%). [α]D296K = +8 (0.01g/mL in CHCl3).

(2S,3S)-4-amino-2,3-bis(hexadecyloxy)1-butanol (13). Compound 12 (189 mg, 0.264 mmol) was dissolved in 3 mL of dichloromethane, to which was added 2.3-dichloro-5,6-dicyano-1,4-benzozquinone (DDQ, 90 mg, 0.396 mmol) and 0.3 mL water. The reaction was stirred at rt for 3 hours. Ethyl ether (150 mL) was added and the solution was extracted with saturated sodium chloride (100 mL), brine (100 mL) and water (100 mL). The organic layer was dried with magnesium sulfate, filtered and concentrated to yield the crude product, which was purified by column chromatography with a gradient solvent system of 10-20% ethyl acetate to hexanes to yield compound 13 as a white solid (115 mg, 75%). [α]D296K = +6. (0.01g/mL in CHCl3).

Tert-Butyl((2S,3S)-2,3-bis(hexadecyloxy)-4-hydroxybutyl)carbamate(15). Compound 13 (176 mg, 0.29 mmol) was dissolved in ethyl acetate (10 mL). While stirring, palladium on carbon (100 mg), triethylamine (200 mg, 0.58 mmol), and di-tert-butyl dicarbonate (Boc2O, 130 mg, 0.58 mmol) were added. The reaction was next stirred under 1 atmosphere of hydrogen (balloon) overnight. The mixture was then filtered through celite and the solvent was removed under reduced pressure. Column chromatography with a gradient solvent system of 20-35% ethyl acetate to hexane afforded compound 15 (168 mg, 92% yield).

[α]D296K = +8.0 (0.01g/mL in CHCl3). 1H NMR (300MHz, CDCl3) δ 3.78-3.76 (m, 1H), 3.64-3.62 (m, 2H), 3.55-3.52 (m, 4H), 3.44-3.42 (m, 2H), 3.18-3.15 (m, 2H), 1.58-1.54 (m, 4H), 1.43 (s, 9H), 1.25 (s, 52H), 0.87 (t, J = 6 Hz, 6H). 13C NMR (500MHz, CDCl3) δ 155.76, 80.36, 79.81, 72.31, 70.66, 60.96, 40.55, 34.08, 31.87, 29.57, 29.30,

4-benzoyl-N-((1-((2S,3S)-2,3-bis(hexadecyloxy)-4-hydroxybutyl)-1H-1,2,3-triazol-4-yl)methyl)benzamidin (2). Azide 13 (45 mg, 76 µmol) was dissolved in 1.5 mL of tetrahydrofuran and 0.5 mL of water was added. To this solution was added alkyne 14 (20 mg, 76 µmol), copper sulfate pentahydrate (19 mg, µmol), and sodium ascorbate (30 mg, 152 µmol), and the reaction was allowed to stir at rt overnight. Next, the crude was extracted from water (50 mL) with dichloromethane (2 x 50 mL), and the organic layers were combined, dried with magnesium sulfate, filtered, and the solvent was removed by rotary evaporation. Purification through column chromatography with gradient elution from 50-100% ethyl acetate to hexanes provided the product as a white solid (52 mg, 80% yield).

\[ \text{H NMR (300MHz, CDCl}_3 \text{)} \delta 7.95 (d, J = 9 Hz, 2H), 7.78-7.81 (m, 5H), 7.62-7.45 (m, 4H), 4.76-4.70 (m, 3H), 4.38-4.31 (m, 1H), 3.89-3.68 (m, 3H), 3.55 (t, J = 6 Hz, 2H), 3.48-3.42 (m, 2H), 3.22-3.17 (m, 1H), 1.63-1.59 (m, 2H), 1.38-1.44 (s, 5, 2H), 1.25 (s, 5, 2H), 0.9 (t, J= 6 Hz, 6H). \]

\[ \text{C NMR (500MHz, CDCl}_3 \text{)} \delta 195.90, 166.46, 144.08, 140.08, 137.25, 136.90, 132.84, 130.07, 128.41, 127.08, 124.06, 78.97, 78.30, 71.90, 71.12, 60.59, 50.93, 35.41, 31.92, 29.36, 25.95, 22.69, 14.13. \]

MALDI-HRMS [M + Na]+ calcd 881.6496; found, 881.6577.

3-benzoyl-N-((2S,3S)-2,3-bis(hexadecyloxy)-4-hydroxybutyl)benzamide (3). Compound 15 (149 mg, 0.21 mmol) was dissolved in dichloromethane and trifluoroacetic acid (TFA, 4 mL) was added. After 3 hrs, the solvent was removed under pressure and the crude was left on the high vacuum for 2 hrs. The crude was then dissolved in dry N,N'-dimethylformamide. 4-dimethylaminopyridine (DMAP, 45 mg, 0.33 mmol), 4-benzoylbenzoic acid (80 mg, 0.33 mmol), and N-methylmorpholine (100 µL, 0.877 mmol) were added and the mixture was stirred overnight. Column chromatography with solvent gradient system of 20-50% ethyl acetate to hexane afforded compound 3 as a white solid (115 mg, 67% yield).

\[ [\alpha]_D^{296K} = +7.6 \text{ (0.01g/mL in CHCl}_3 \text{)}. \]

\[ \text{H NMR (500MHz, CDCl}_3 \text{)} \delta 7.90-7.85 (m, 3H), 7.80 (d, J = 9 Hz, 2H), 7.60 (t, J = 6 Hz 1H), 7.50 (t, J = 6 Hz 2H), 7.16 (s, J = 6 Hz 1H), 4.00-3.95 (m, 1H), 3.90-3.85 (m, 1H), 3.75-3.70 (m, 2H), 3.65-3.60 (m, 2H), 3.59-3.50 (m, 3H), 3.40-3.30 (m, 2H), 2.30(t,J = 6 Hz 1H), 2H), 1.65-1.50 (m, 4H), 1.40-1.20 (m, 52H), 0.9 (t, J= 6 Hz, 6H). \]

\[ \text{C NMR (500MHz, CDCl}_3 \text{)} \delta 195.84, 166.37, 139.98, 138.08, 137.06, 132.78, 129.94, 128.28, 126.46, 80.27, 70.79, 61.10, 39.07, 31.91, 29.68, 29.34, 29.25, 26.25, 22.67, 14.09. \]

MALDI-HRMS [M + H]+ calced for 778.6974; found 778.6959. MALDI-HRMS.

N-((2S,3S)-2,3-bis(hexadecyloxy)-4-hydroxybutyl)-5-(3-methyl-3H-diazirin-3-yl)pentanamide (4). Compound 15 (162 mg, 0.21 mmol) was dissolved in dichloromethane and while stirring trifluoroacetic acid (TFA, 4 mL) was added. After 3 hours, the solvent was removed under pressure and the crude was left on the high vacuum for 2 hrs. The crude was then dissolved in dry N,N'-dimethylformamide. 4-Dimethylaminopyridine DMAP (45 mg, 0.33 mmol), 5-(3-methyl-3H-diazirin-3-yl)pentanoic acid (16, 80 mg, 0.33 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 70 mg, 0.33 mmol), and N-methylmorpholine (100 µL, 0.877 mmol) were added and the mixture was stirred overnight. Column chromatography with solvent gradient system of 30-50% ethyl acetate to hexane afforded compound 3 as a brownish-white solid (121mg, 70% yield).

\[ \text{H NMR (500MHz, CDCl}_3 \text{)} \delta 7.18 (s, J = 6 Hz 1H), 4.40-4.30 (m, 1H), 4.20-4.10 (m, 1H), 3.93-3.87 (m, 2H), 3.76-3.66 (m, 2H), 3.64-3.60 (m,2H), 3.59-3.50 (m, 3H),3.40-3.30 (m, 2H), 2.30(t,J = 6 Hz 1H), 2H), 1.65-1.50 (m, 6H), 1.35-1.20 (m, 56H), 1.02(s, J = 6 Hz, 3H), 0.98-0.80 (m, 6H). \]

\[ \text{C NMR (500MHz, CDCl}_3 \text{)} \delta 172.52, 83.56, 79.05, 75.84, 71.94, 71.02, 69.72, 62.61, 39.76, 33.96, 33.65, 32.04, 29.74, 29.44, 29.13, 25.84, 22.94, 20.11 19.42, 14.09. \]

MALDI-HRMS [M + H2O]+ calced for 801.032; found 801.060.