Supporting Information

Mixed Squaramide Thioesters as Phosphate Group Analogues for Non-Competitive Antagonists of the Phospholipid-Sensing G Protein-Coupled Receptor GPR55

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Content

1. Supporting Figures

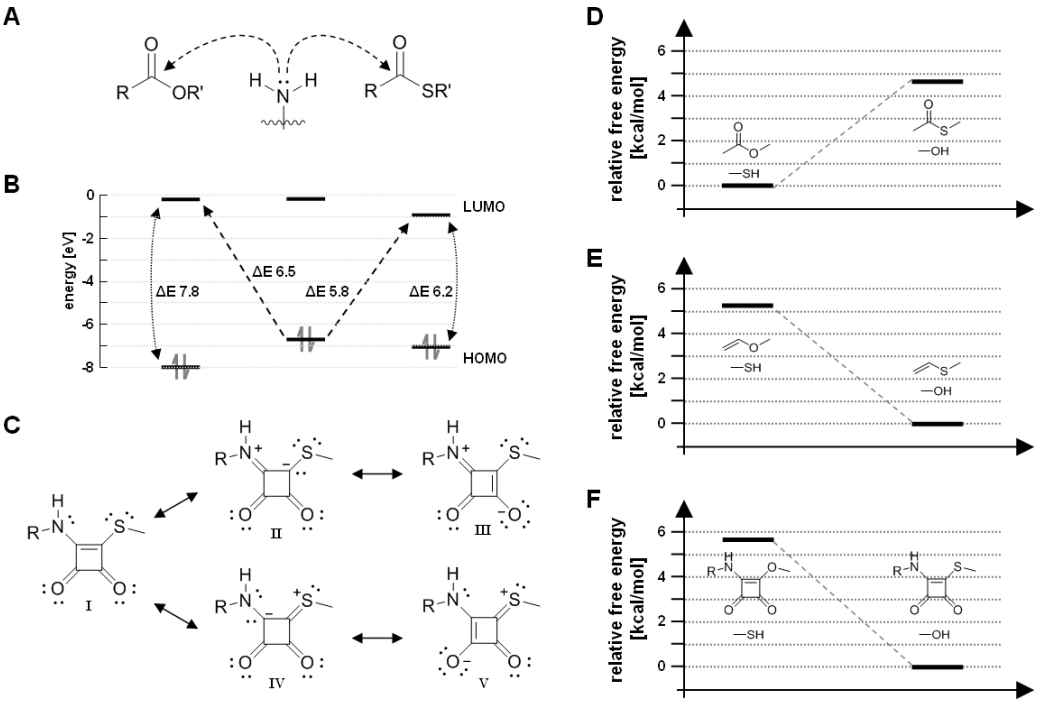


Figure S1: Schematic representation of acylester and acylthioester model substrates (A) and their respective relative HOMO and LUMO energies (B) in aqueous media obtained through DFT calculations with B3LYP/aug-cc-pVTZ basis set. Representative resonance structures of squareamide thioesters (C). Relative free energy of acylester and acylthioester (D), of vinyl ether and vinyl thioether (vinyl sulphide) (E), and of mixed squaramide ester and mixed squaramide thioester model substrates (F).

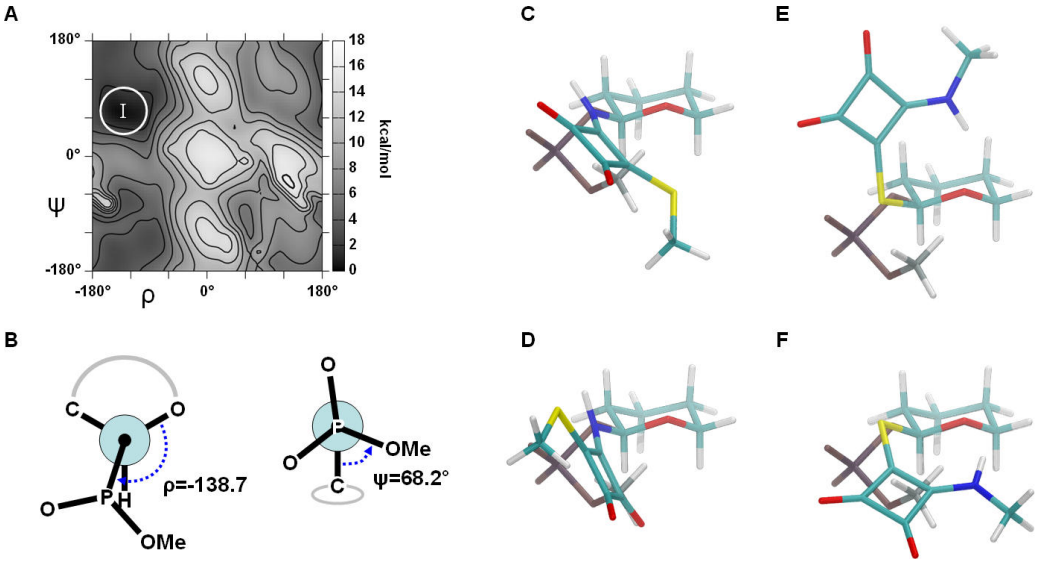


Figure S2: Energy landscape based on DFT calculations at the MP2/6-31g(d) level of an equatorial tetrahydropyranephosphomethan (TPMe) model compound (A). Newman projection of the ρ and ψ angles depicting the lowest energy conformation of TPMe (B). Superposition of TPME (grey) and type 1 model at its primary (C) and secondary (D) minima. Superposition of TPMe (grey) and type 2 model at its primary (E) and secondary (F) minima. Area I, lowest energy conformation; carbon, cyan; oxygen, red; hydrogen, white; nitrogen, blue; sulphur, yellow; phosphate, purple.

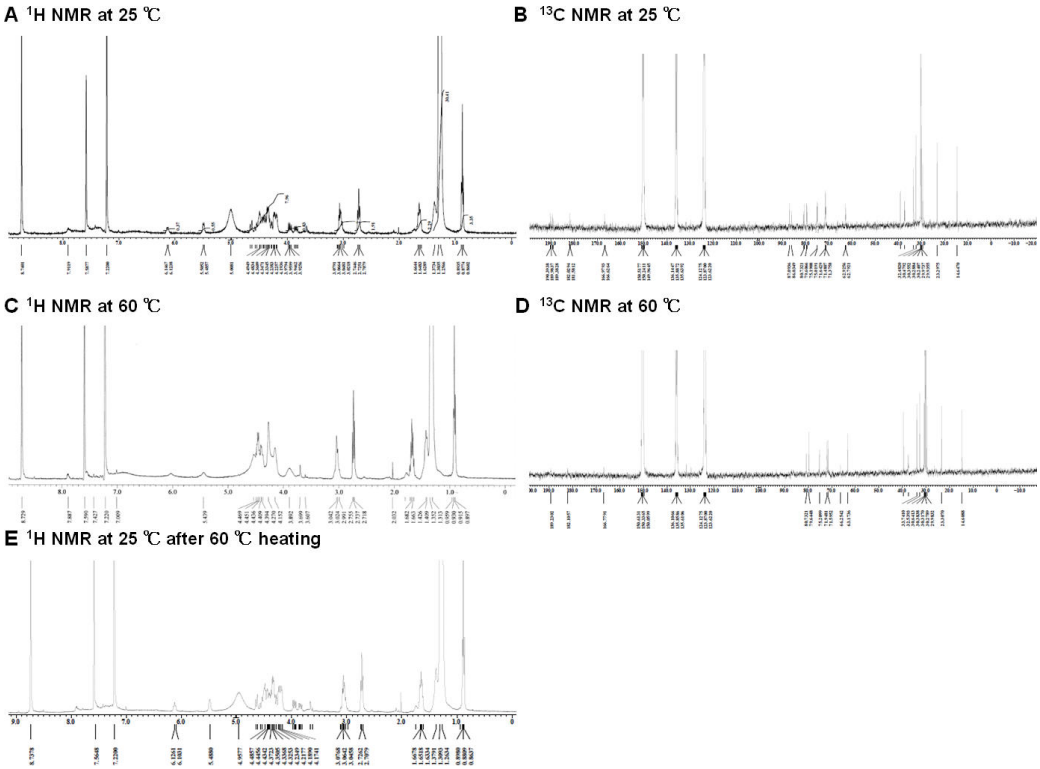


Figure S3: Confirmation of the presence of rotamers in **1a** at 25°C. (A-D) ^{1}H and ^{13}C NMR of **1a** at 25°C and 60°C in d_6-pyridine as indicated. (E) ^{1}H NMR of **1a** at 25°C following heating to 60°C, confirming stability of **1a** at elevated temperature.

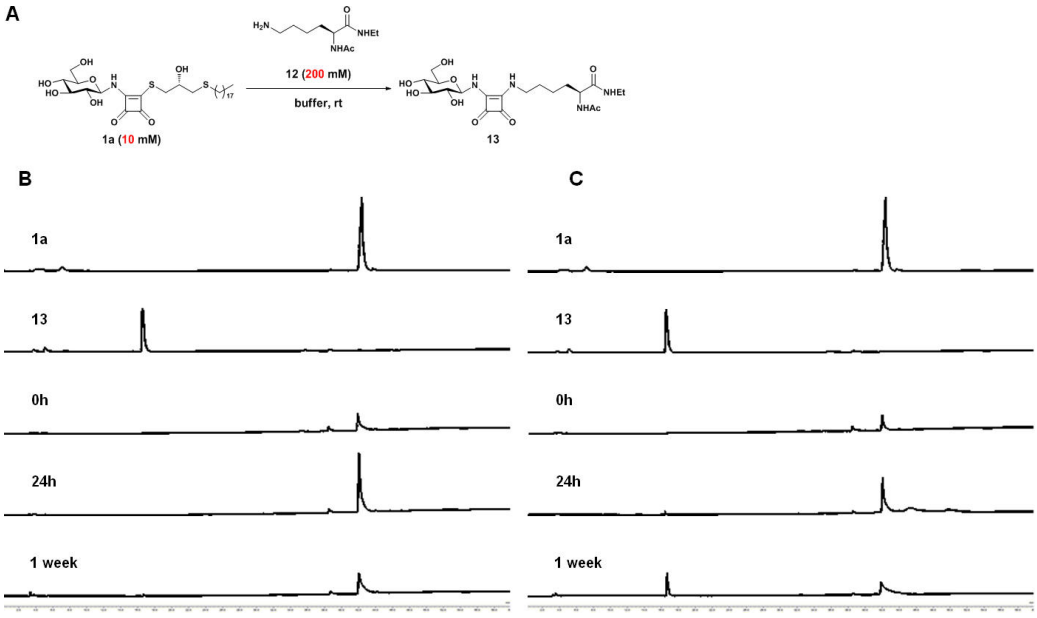


Figure S4: Susceptibility of the mixed squarylamide thioester moiety of **1a** towards nucleophilic attack by soluble, capped lysine homologue **12** and its hydrolytic stability. (A) Reaction scheme. (B) HPLC traces at indicated time points under neutral conditions, pH=7.16. (C) HPLC traces at indicated time points under midly alkaline conditions, pH=9.08.

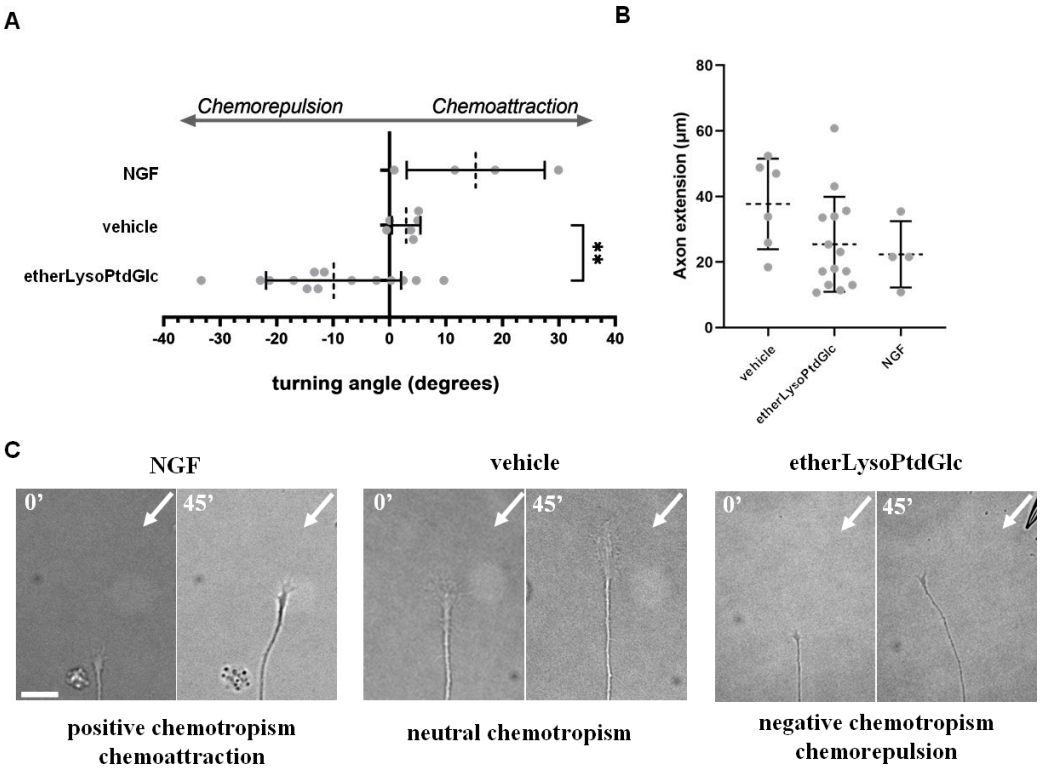


Figure S5: Chemotropic response of primary cultured sensory neurons to a microscopic concentration gradient of guidance cue (NGF or etherLysoPtdGlc) or vehicle control. (A) Observed chemotropic responses corroborate previous reports of NGF-induced chemoattraction and etherLysoPtdGlc-induced chemorepulsion. (B) Axon extension during assay: no statistically significant difference in axon extension was observed between groups. Each grey circle represents an individual axon tested; broken line and bars indicate mean±SD. (C) Representative images of the beginning and end of the assay. Numbers indicate time in minutes elapsed after establishment of microscopic concentration gradient, white arrow, the origin of guidance cue gradient; bar, 20µm.

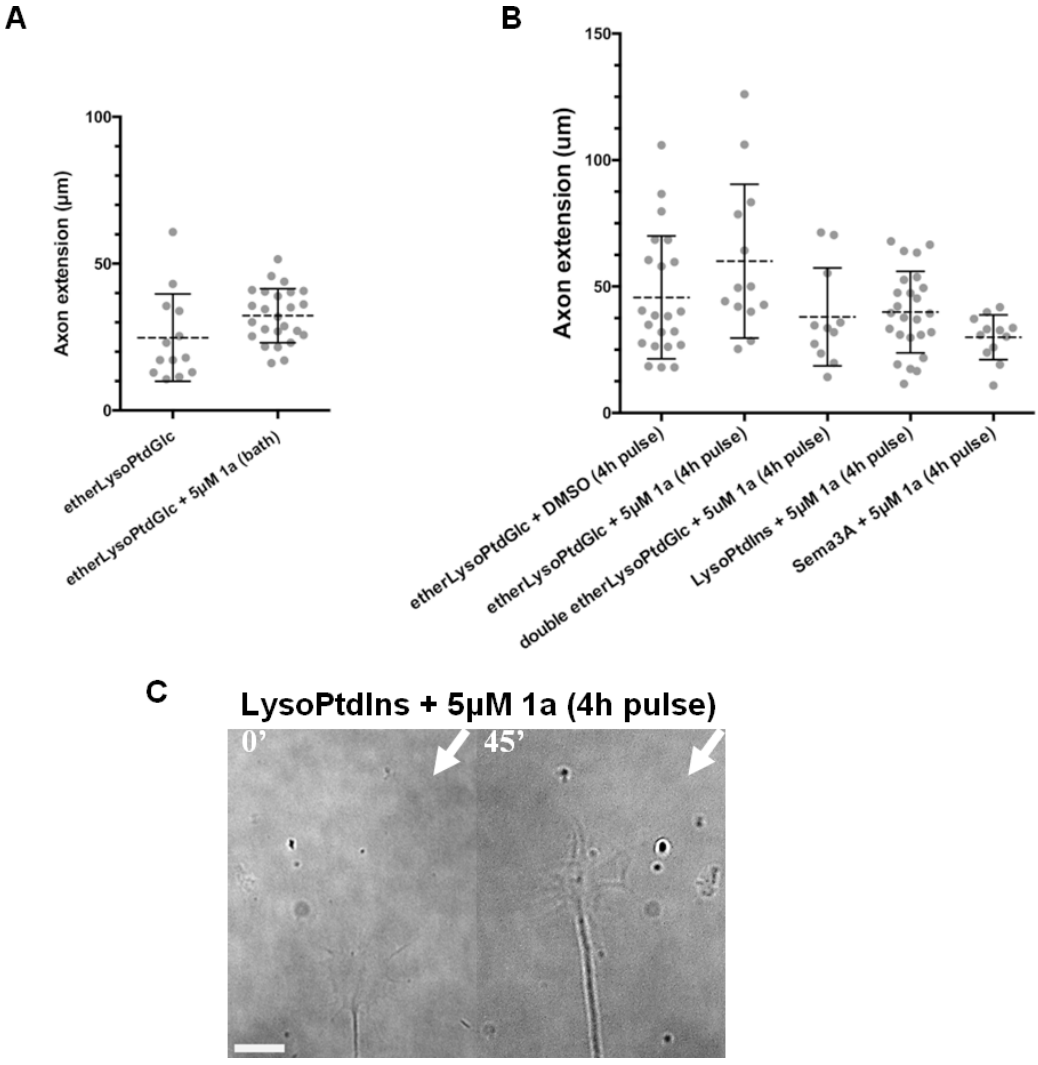


Figure S6: Axon extension in presence of **1a** during acute (A) or sustained (B) treatment assay; each grey circle represents an individual axon tested; broken line and bars indicate mean±SD. (C) Representative images of the beginning and end of the assay. Numbers indicate time elapsed in minutes after establishment of microscopic concentration gradient; white arrow, the origin of guidance cue gradient; bar, 20µm.

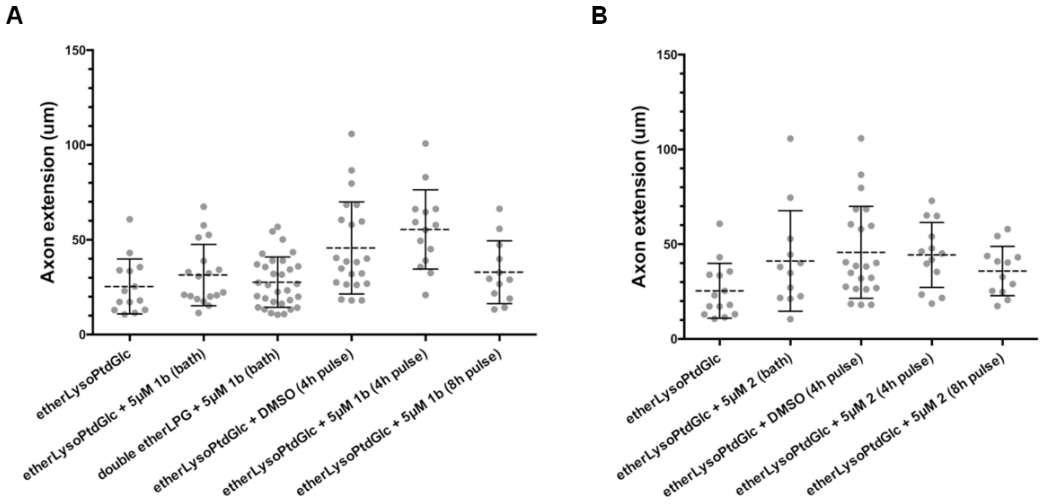
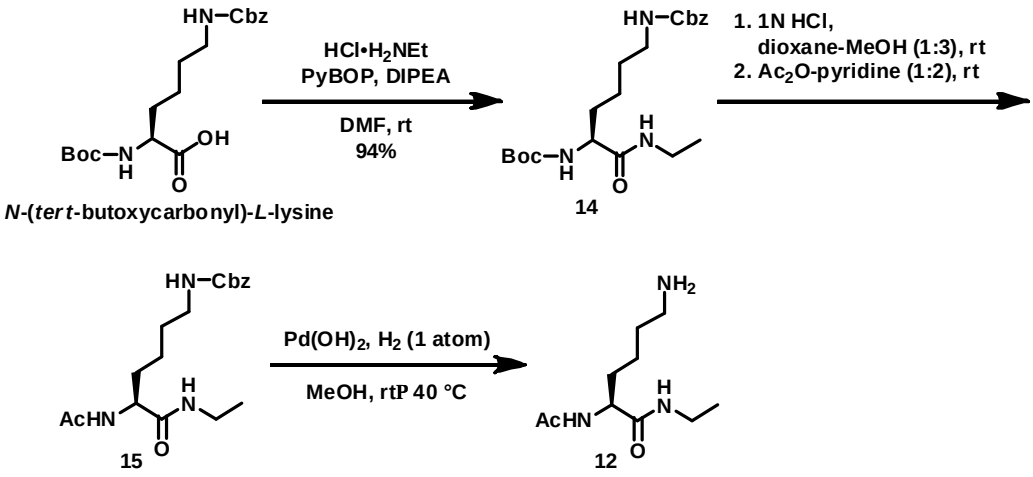


Figure S7: Axon extension in presence of **1b** (A) or **2** (B) during acute and sustained treatment assay. No statistically significant difference in axon extension was observed in either treatment regime. The datasets used for etherLysoPtdGlc and etherLysoPtdGlc + DMSO (4h pulse) are the same as used in Fig. 6A and Fig. 6B, respectively. Each grey circle represents an individual axon tested; broken line and bars indicate mean±SD.



Scheme S1: Synthesis of *N*-Ac-Lys-NHEt (**12**).

2. Materials and Methods

2.1 General synthetic procedures

Air and/or moisture sensitive reactions were carried out under argon atmosphere with anhydrous solvents. Reactions were monitored by thin-layer chromatography (TLC) (Kieselgel 60 F254, EM Science). Compounds were detected by UV light and/or Hanessian's stain and purified by column chromatography (silica gel 60, 40-100 mesh, EM Science). Solvents were removed lower than 40 ºC under reduced pressure. Synthesised compounds were analyzed by ^{1}H NMR and ^{13}C NMR (JEOL ECX 400 spectrometer). ^{1}H NMR analyses were conducted with $CDCl_3$, CD_3OD or pyridine-d_5 referenced to $CHCl_3$ at 7.26 ppm, to CHD_2OD at 3.31 ppm or to pyridine at 7.22 ppm, respectively. ^{13}C NMR analyses were referenced to $CDCl_3$ at 77.0 ppm, CD_3OD at 49.00 or pyridine-d_5 at 123.87 ppm. Synthesised compounds were assigned by standard 2D experiments. High-resolution mass spectra were recorded on a Thermo Fisher Scientific Orbitrap XL. Reagents were purchased from Kanto Chemicals Co. Inc., Tokyo Chemical Industries Co., Ltd., FUJIFILM Wako Pure Chemical Co., Aldrich Chemical Co. and Avanti Polar Lipids, Inc unless stated otherwise.

2.2 Synthetic procedures

2.2.1 (*S*)-1-(octadecylthio)-3-(trityloxy)propan-2-ol (4)

To a solution of **3** (386.0 mg, 1.22 mmol) and *n*-octadecanethiol (420.0 mg, 1.47 mmol) in THF (6.0 mL) and ethanol (6.0 mL) under argon atmosphere on ice bath was added sodium ethoxide (83.0 mg, 1.22 mmol). The reaction mixture was stirred at ambient temperature for 17.5 h and diluted with ethyl acetate, washed with brined three times and dried over Na_2SO_4. After filtration and evaporation under reduced pressure, the crude product was purified by column chromatography (silicagel, hexane / ethyl acetate = 20 / 1 to 8 / 1) to afford **4** (white solid, 653 mg, 89% yield) $[\alpha]_D^{26}$ = 5.4 (c 1.0 in $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.50 (d, *J* = 7.2 Hz, 6H), 7.35 (t, *J* = 7.2 Hz, 6H), 7.28 (t, *J* = 6.7 Hz, 3H), 3.89 (m, 1H), 3.29 (m, 2H), 2.82-2.62 (m, 3H), 2.51 (t, *J* = 7.2 Hz, 2H), 1.60 (m, 2H), 1.32 (m, 30H), 0.934 (t, *J* = 7.2 Hz, 3H) ; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 143.73, 128.60, 127.81, 127.04, 86.71, 69.26, 66.29, 36.29, 32.40, 31.89, 29.66, 29.57, 29.49, 29.32, 29.20, 28.80, 22.66, 14.10 ; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{40}H_{58}O_2SNa$ 625.4055, found 625.4052.

2.2.2 (*S*)-3-(octadecylthio)propane-1,2-diol (5)

To a solution of **4** (160.0 mg, 0.265 mmol) in methanol (2.0 mL) and DCM (2.0 mL) was added 4N HCl-dioxane (1.3 mL). The reaction mixture was stirred at ambient temperature for 3 h. After evaporation under reduced pressure, the crude product was purified by column chromatography (silicagel, hexane / ethyl acetate = 4 / 1 to ethyl acetate) to afford **5** (white solid, 91 mg, 95% yield). $[\alpha]_D^{26}$ = 22.8 (c 1.0 in $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.77 (m, 2H), 3.55 (m, 1H), 2.71-2.58 (m, 2H), 2.53 (t, *J* = 7.6 Hz, 3H), 1.58 (m, 2H), 1.41-1.21 (m, 30H), 0.873 (t, *J* = 6.7 Hz, 3H) ; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 69.63, 65.40, 35.82, 32.29, 31.90, 29.68, 29.57, 29.49, 29.34, 29.19, 28.83, 22.67, 14.10 ; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{21}H_{44}O_2SNa$ 383.2954, found 383.2953.

2.2.3 (*S*)-2-hydroxy-3-(octadecylthio)propyl 2,4,6-trimethylbenzenesulfonate (6)

To a solution of **5** (90.0 mg, 0.250 mmol), DMAP (3.1 mg, 0.0250 mmol) and triethylamine (70 µL, 0.499 mmol) in DCM (2.5 mL) on ice bath was added 2,4,6-triisopropylbenzene sulfonic chloride (91.0 mg, 0.299 mmol). The reaction mixture was stirred at ambient temperature for 13 h. The reaction was quenched with methanol and the mixture was diluted with ethyl acetate, washed with brine three times and dried over Na_2SO_4. After filtration and evaporation under reduced pressure, the crude product was purified by column chromatography (silicagel, hexane / ethyl acetate = 30 / 1 to 8 / 1) to afford **6** (white solid, 90 mg, 58% yield). $[\alpha]_D^{26}$ = 1.6 (c 1.0 in $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.19 (s, 2H), 4.12 (m, 4H), 3.97 (m, 1H), 2.91 (m, 1H), 2.77-2.57 (m, 2H), 2.51 (t, *J* = 7.2 Hz, 2H), 1.56 (m, 2H), 1.38-1.21 (m, 48H), 0.873 (t, *J* = 6.7 Hz, 3H) ; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 153.91, 150.86, 128.91, 123.81, 71.00, 67.89, 35.54, 34.22, 32.51, 31.89, 29.66, 29.63,

29.55, 29.47, 29.32, 29.16, 28.75, 24.70, 23.49, 22.66, 14.09 ; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{36}H_{66}O_4S_2Na$ 649.4295, found 649.4294.

2.2.4 (*R*)-S-(2-hydroxy-3-(octadecylthio)propyl) ethanethioate (7)

To a solution of **6** (90.0 mg, 0.144 mmol) and potassium thioacetate (25.0 mg, 0.215 mmol) in DMF (1.4 mL) was added 18-crown-6 ether (46 µL, 0.215 mmol). The reaction mixture was stirred at ambient temperature for 20 h and diluted with ethyl acetate, washed with brine three times and dried over Na_2SO_4. After filtration and evaporation under reduced pressure, the crude product was purified by column chromatography (silicagel, hexane / ethyl acetate = 8 / 1) to afford **7** (white solid, 51 mg, 84% yield). $[\alpha]_D^{26}$ = -0.20 (c 1.0 in $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.81 (m, 1H), 3.18 (m, 1H), 3.01 (m, 1H), 2.88 (m, 1H), 2.74 (m, 1H), 2.58-2.49 (m, 3H), 2.36 (s, 3H), 1.56 (m, 2H), 1.38-1.21 (m, 30H), 0.867 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 196.04, 68.75, 38.46, 34.63, 32.35, 31.88, 30.50, 29.66, 29.55, 29.47, 29.32, 29.16, 28.77, 22.65, 14.08 ; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{23}H_{46}O_2S_2Na$ 441.2831, found 441.2845.

2.2.5 (*S*)-1-mercapto-3-(octadecylthio)propan-2-ol (8)

To a solution of **7** (40.0 mg, 0.0955 mmol) in DCM (1.5 mL) and methanol (1.5 mL) was added 4N HCl-dioxane (1.0 mL) under argon atmosphere. The reaction mixture was stirred at ambient temperature for 4 h. After evaporation under reduced pressure, the crude product was purified by column chromatography (silicagel, hexane / ethyl acetate = 8 /1) to afford **8** (colorless oil, 35 mg, 97% yield). $[\alpha]_D^{26}$ = -13.0 (c 1.0 in $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.74 (m, 1H), 2.79-2.62 (m, 4H), 2.54 (t, *J* = 7.2 Hz, 2H), 1.62-1.56 (m, 2H), 1.51 (t, *J* = 8.5 Hz, 1H), 1.39-1.22 (m, 30H), 0.877 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 70.35, 37.84, 32.48, 31.92, 30.36, 29.68, 29.58, 29.49, 29.35, 29.20, 28.81, 22.68, 14.12 ; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{21}H_{44}OS_2Na$ 399.2726, found 399.2713.

2.2.6 3-ethoxy-4-(*N*-β-D-galactopyranosylamino)cyclobut-3-ene-1,2dione (9b)

D-galactose (700 mg, 3.89 mmol) and ammonium bicarbonate (308 mg, 3.90 mmol) were suspended in aq. NH_3 (19 mL) and the resultant mixture was stirred at 42 ℃ for 18 h. Following degassing under reduced pressure, the remaining mixture was lyophilised. The crude residue was suspended in ethanol (8.5 mL), treated with diethyl squarate (862 µL, 5.83 mmol) and DIPEA (339 µL, 1.95 mmol) and stirred at ambient temperature for 14 h. After evaporation under reduced pressure, the crude product was purified by column chromatography (silica gel, $CHCl_3$ / methanol = 10/1 to 5/1) to afford **9b** (yellow amorphous, 560 mg, 48% yield). $[\alpha]_D^{26}$ = -3.70 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 4.99 (br s, 1H), 4.75 (q, *J* = 7.1 Hz, 2H), 3.90 (d, *J* = 3.1 Hz, 1H), 3.90-3.60 (m, 4H), 3.54 (d, *J* = 7.1 Hz, 1H), 1.44 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 189.58, 179.43, 175.31, 86.42, 85.73, 78.49, 75.43, 72.01, 71.02, 70.40, 69.41, 62.45, 16.07 ; HRMS (ESI) m/z $[M+H]^+$ calculated for $C_{12}H_{18}NO_8$ 304.1027, found 304.1027.

2.2.7 3-(((*R*)-2-hydroxy-3-(octadecylthio)propyl)thio)-4-(*N*-β-D-glucopyranosylamino)cyclobut-3-ene-1,2dione (1a)

To a solution of 3-ethoxy-4-(*N*-β-D-glucopyranosylamino)cyclobut-3-ene-1,2dione (**9a**, 16.6 mg, 0.0547 mmol) and **8** (20.6 mg, 0.0547 mmol) in ethanol was added DIPEA (9.50 µL, 0.0547 mmol) and the resulting mixture was stirred at ambient temperature overnight. After evaporation under reduced pressure, the crude product was purified by column chromatography (silicagel, $CHCl_3$ / methanol = 10 /1) to afford **1a** (11.0 mg, 0.0372 mmol). $[\alpha]_D^{26}$ = 11.6 (c 0.5 in pyridine). ^{1}H NMR (pyridine-d_5, 400 MHz): δ 6.15 (s, 0.4 H), 5.49 (d, *J* = 7.6 Hz, 0.6 H), 4.57 (t, *J* = 12.1 Hz, 1H), 4.51-4.08 (m, 7H), 3.97-3.82 (m, 1H), 3.07 (t, *J* = 7.1 Hz, 2H), 2.73 (t, *J* = 6.7 Hz, 2H), 1.65 (t, *J* = 7.6 Hz, 2H), 1.43-1.20 (m, 30H), 0.877 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (pyridine-d_5, 100 MHz): δ 190.39, 189.98, 189.38, 189.00, 182.03, 181.58, 166.98, 166.63, 87.09, 86.03, 80.73, 79.61, 75.19, 75.02, 71.64, 71.48, 71.38, 62.93, 62.79, 39.17, 37.45, 37.27, 33.48, 32.48, 30.48, 30.36, 30.29, 29.97, 29.54, 23.30, 14.65 ; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{31}H_{56}NO_8S_2$ 634.3442, found 634.3443.

2.2.8 3-(((*R*)-2-hydroxy-3-(octadecylthio)propyl)thio)-4-(*N*-β-D-galactopyranosylamino)cyclobut-3-ene-1,2dione (1b)

To a solution of 3-ethoxy-4-(*N*-β-D-galactopyranosylamino)cyclobut-3-ene-1,2dione (**9b**, 18.8 mg, 0.0620 mmol) and **8** (23.4 mg, 0.0620 mmol) in ethanol was added DIPEA (10.8 µL, 0.0620 mmol) and the resulting mixture was stirred at ambient temperature overnight. After evaporation under reduced pressure, the crude product was purified by column chromatography (silicagel, $CHCl_3$ / methanol = 10 /1) to afford **1b** (11.0 mg, 0.0372 mmol). $[\alpha]_D^{26}$ = -12.2 (c 0.5 in pyridine). ^{1}H NMR (pyridine-d_5, 400 MHz): δ 6.08 (s, 0.5H), 5.44 (d, *J* = 10.3 Hz, 0.5H), 4.79 (t, *J* = 9.4 Hz, 1H), 4.72-4.19 (m, 7H), 3.08 (m, 2H), 2.73 (m, 2H), 1.66 (m, 2H), 1.49-1.22 (m, 30H), 0.876 (t, *J* = 7.2 Hz, 3H) ; ^{13}C NMR (pyridine-d_5, 100 MHz): δ 190.20, 189.43, 189.18, 181.90, 181.38, 168.14, 166.35, 87.94, 86.64, 79.37, 79.07, 76.19, 72.50, 72.21, 71.45, 70.63, 66.19, 62.85, 39.19, 37.58, 37.26, 33.48, 32.48, 30.49, 30.37, 30.29, 29.97, 29.55, 26.27, 23.30, 14.65 ; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{31}H_{56}NO_8S_2$ 634.3442, found 634.3429

2.2.9 (*S*)-3-ethoxy-4-((2-hydroxy-3-(octadecylthio)propyl)amino)cyclobut-3-ene-1,2dione (11a)

To a suspension of (*S*)-1-amino-3-(octadecylthio)propan-2-ol (40.0 mg, 0.111 mmol), DIPEA (19.0 µL, 0.111 mmol) in ethanol (1.1 mL) was added diethhylsquarate (33.0 µL, 0.222 mmol). The mixture was stirred at ambient temperature for 45 min. After evaporation under reduced pressure, the crude product was purified by column chromatography (silicagel, chloroform / methanol = 30 / 1 to 10 / 1) to afford **11a** (white solid, 41 mg, 76% yield). $[\alpha]_D^{26}$ = 8.2 (c 1.0, $CHCl_3$). ^{1}H NMR (pyridine-d_5, 400 MHz): δ 10.48 (br s, 0.6H), 10.04 (br s, 0.4H), 4.75 (q, *J* = 7.0 Hz, 1.2H), 4.66 (q, *J* = 7.0 Hz, 0.8Hz), 4.40-3.78 (m, 3H), 2.99 (m, 2H), 2.70 (t, *J* = 7.4 Hz, 2H), 1.65 (m, 2H), 1.36 (t, *J* = 7.0 Hz, 3H), 1.35-1.26 (m, 28H), 0.88 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (pyridine-d_5, 100 MHz): δ 191.13, 190.87, 184.07, 183.86, 178.21, 178.04, 174.67, 174.29, 71.52, 71.25, 69.55, 69.45, 50.80, 50.40, 37.72, 33.46, 32.49, 30.47, 30.36, 30.28, 30.21, 29.97, 29.91, 29.51, 23.30, 16.31, 16.04, 14.64; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{31}H_{56}NO_8S_2$ 634.3442, found 634.3425.

2.2.10 (*S*)-3-((2-hydroxy-3-(octadecylthio)propyl)amino)-4-phenoxycyclobut-3-ene-1,2dione (11b)

To a solution of diphenylsquarate (29.0 mg, 0.109 mmol) and DIPEA (19.0 µL, 0.109 mmol) in dry dichloromethane (1.0 mL) was added (*S*)-1-amino-3-(octadecylthio)propan-2-ol (33.0 mg, 0.109 mmol). The resultant mixture was stirred at ambient temperature for 1 h and subsequently purified directly by column chromatography (silicagel, hexane / ethylacetate = 2 / 1 to 1 / 1) to afford **11b** (pale yellow solid, 32.0 mg, 62% yield). $[\alpha]_D^{26}$ = -5.1 (c 0.5 in pyridine). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.41 (m, 2H), 7.30-7.20 (m, 3H), 6.26 (br s, 0.5H), 5.67 (br s, 0.5H), 4.10-3.80 (m, 3H), 3.58 (m, 1H), 2.90 (br s, 1H), 2.75 (d, *J* = 11 Hz, 1H), 2.53 (t, *J* = 7.2 Hz, 3H), 1.57 (m, 2H), 1.40-1.20 (m, 30H), 0.879 (t, *J* = 6.8 Hz, 3H) ; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 190.18, 189.81, 181.27, 180.96, 174.09, 173.49, 173.24, 154.55, 154.30, 153.25, 153.02, 129.94, 129.76, 125.98, 125.78, 118.18, 68.31, 68.21, 48.96, 48.66, 36.75. 32.49, 32.23, 31.91, 29.68, 29.64, 29.58, 29.50, 29.35, 29.19, 28.80, 22.68, 14.11; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{31}H_{49}O_4SNa$ 554.3275, found 554.3274.

2.2.11 3-(((*S*)-2-hydroxy-3-(octadecylthio)propyl)thio)-4-(*S*-β-D-galactopyranosylthio)cyclobut-3-ene-1,2dione (2)

To a solution of **11b** (26.0 mg, 0.0547 mmol) and 1-thio-β-D-glucoside (10.7 mg, 0.0547 mmol) in dimethylsulfoxide (0.547 mL) was added DIPEA (9.50 µL, 0.0547 mmol). The mixture was stirred at ambient temperature for 16.5 h and the reaction mixture was subsequently purified directly by column chromatography (silicagel, chloroform / methanol = 50 / 1 to 10 / 1) to afford **2** (white solid, 26.5 mg, 84% yield). $[\alpha]_D^{26}$ = -32.5 (c 0.50 in pyridine). ^{1}H NMR (pyridine-d_5, 400 MHz): δ 10.84 (br s, 0.2H), 9.55 (m, 0.6H), 6.77 (d, *J* = 10.0 Hz, 0.3H), 5.78 (d, *J* = 9.8 Hz, 0.7H), 4.52-4.09 (m, 9H), 3.02-2.93 (m, 1H), 2.93 (t, *J* = 6.4 Hz, 1H), 2.71 (t, *J* = 7.4 Hz, 0.6H), 2.66 (t, *J* = 7.4 Hz, 1.4H), 1.58 (m, 2H), 1.28 (m, 30H), 0.88 (t, *J* = 7.0 Hz, 3H) ; ^{13}C NMR (pyridine-d_5, 100 MHz): δ 190.67, 189.84, 188.98, 188.67, 182.14, 181.69, 162.36, 161.98, 124.36, 124.12, 123.87, 123.62, 123.32, 85.44, 83.97, 83.34, 82.96, 80.69, 80.18, 75.41, 75.02, 71.59, 71.38, 70.93,

62.90, 62.06, 51.47, 50.70, 37.94, 37.74, 33.55, 33.44, 32.47, 30.45, 30.40, 30.35, 30.28, 30.22, 29.96, 29.91, 29.51, 23.29, 14.64 ; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{31}H_{55}NO_8S_2Na$ 656.3261, found 656.3262.

2.2.12 (*S*)-benzyl *tert*-butyl (6-(ethylamino)-6-oxohexane-1,5-diyl)dicarbamate (14)

To a solution of *N*-(tert-butoxycarbonyl)-*L*-lysine (500.0 mg, 1.31 mmol), DIPEA (0.570 mL, 3.28 mmol) and ethylamine hydrochloride (161.0 mg, 1.97 mmol) was added PyBOP (1025.0 mg, 1.97 mmol). The mixture was stirred at ambient temperature for 21 h and subsequently diluted with ethylacetate, washed with brine three times and dried over Na_2SO_4. After filtration and evaporation under reduced pressure, the crude residue was purified by column chromatography (silicagel, hexane / ethylacetate = 1 / 1 to 1 / 2) to afford **14** (white solid, 501.8 mg, 94% yield). $[\alpha]_D^{26}$ = -12.6 (c 1.0 in $CHCl_3$). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.34-7.28 (m, 5H), 5.06 (s, 2H), 3.94 (m, 1H), 3.20 (m, 2H), 3.11 (t, J = 6.8 Hz, 2H), 1.71-1.38 (m, 15H), 1.10 (t, J = 7.2 Hz, 3H) ; ^{13}C NMR (CD_3OD, 100 MHz): δ 174.93, 158.94, 157.80, 138.44, 129.44, 128.93, 128.75, 80.56, 67.32, 56.09, 41.41, 35.20, 33.14, 30.49, 28.69, 24.07, 14.80; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{21}H_{33}N_3O_5Na$ 430.2312, found 430.2313.

2.2.13 (*S*)-benzyl (5-acetamido-6-(ethylamino)-6-oxohexyl)carbamate (15)

To a solution of **14** (444.0 mg, 1.09 mmol) in methanol (6.0 mL) was added 4N HCl-dioxane (2.0 mL). The mixture was stirred at ambient temperature for 1 h and subsequently the solvent was removed by evaporation under reduced pressure. The crude residue was dissolved in pyridine (2.0 mL) and treated with acetic anhydride (1.0 mL). The mixture was stirred at ambient temperature for 28 h. After evaporation under reduced pressure, the crude residue was purified by column chromatography (silicagel, chloroform / methanol = 10 / 1 to methanol) to afford **15** in two steps (brown solid, 171.4 mg, 45% yield). $[\alpha]_D^{26}$ = -18.2 (c 1.0 in $CHCl_3$). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.34-7.28 (m, 5H), 5.06 (s, 2H), 4.23 (dd, *J* = 5.5, 8.5 Hz, 1H), 3.19 (m, 2H), 3.11 (t, *J* = 6.8 Hz, 2H), 1.97 (s, 3H), 1.78-1.71 (m, 1H), 1.67-1.58 (m, 1H), 1.50 (m, 2H), 1.38 (m, 2H), 1.10 (t, *J* = 7.2 Hz, 3H) ; ^{13}C NMR (CD_3OD, 100 MHz): δ 174.21, 173.25, 158.90, 138.43, 129.44, 128.92, 128.73, 67.29, 54.89, 41.44, 35.35, 32.83, 30.49, 24.06, 22.47, 14.80; HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{18}H_{27}N_3O_4Na$ 372.1894, found 372.1891.

2.2.14 (*S*)-2-acetamido-6-amino-*N*-ethylhexanamide (12)

To a solution of **15** (148.0 mg, 0.424 mmol) in methanol (4.0 mL) under argon atmosphere was added $Pd(OH)_2$ (15.0 mg). After the mixture was stirred under hydrogen atmosphere (1 atm) at ambient temperature for 2.5 h, $Pd(OH)_2$ (30.0 mg) was added and then the mixture was stirred at ambient temperature for an additional 3 h. Subsequently, the mixture was stirred at 40 °C for 16 h. Following celite filtration and washing, the resultant mixture was evaporated under reduced pressure. The crude residue was purified by column (silicagel, chloroform / methanol = 10 / 1 containing 0.1% (v/v) Et_3N to methanol containing 0.1% (v/v) Et_3N) to afford **12** (yellow oil, 40 mg, 44% yield). $[\alpha]_D^{26}$ = -11.1 (c 1.0 in MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 4.25 (dd, *J* = 5.7, 8.6 Hz, 1H), 3.20 (q, *J* = 7.2 Hz, 2H), 2.73 (t, *J* = 7.3 Hz, 2H), 1.99, (s, 3H), 1.81-1.34 (m, 6H), 1.11 (t, *J* = 7.2 Hz, 3H) ; ^{13}C NMR (CD_3OD, 100 MHz): δ 174.13, 173.32, 54.85, 41.64, 35.24, 32.88, 31.64, 24.14, 22.48, 14.79; HRMS (ESI) m/z $[M+H]^+$ calculated for $C_{10}H_{22}N_3O_2$ 216.1707, found 216.1708

2.3 *In vitro* susceptibility of mixed squarylamide thioester 1a towards nucleophilic attack

2.3.1 Under neutral conditions

A solution of **12** (6 mL, 1M in 300 mM phosphate buffer (pH 7.16)) was diluted with phosphate buffer (18 µL, 300 mM, (pH 7.16)). Subsequently phosphate buffer (3 µL, 600 mM (pH 7.16)) and **1a** (3 µL, 100 mM in DMSO) were added and the resulting mixture was stirred at ambient temperature. Aliquots (5 µL) were removed at the desired time points and immediately quenched with aqueous TFA solution (15 µL, 0.1% aq.) and subjected to HPLC analysis.

2.3.2 Under mild alkaline conditions

A solution of **13** (6 µL, 1M in 300 mM Tris-HCl buffer (pH 9.08)) was diluted with Tris-HCl buffer (18 µL, 300 mM, (pH 9.08)). Subsequently Tris-HCl buffer (3 µL, 600 mM (pH 9.08)) and **1a** (3 µL, 100 mM in DMSO) were

added and the resulting mixture was stirred at ambient temperature. Aliquots (5 µL) were removed at the desired time points and immediately quenched with aqueous TFA solution (15 µL, 0.1% aq.) and subjected to HPLC analysis.

2.3.3 HPLC analysis

The quenched mixture was analyzed using a C4 reverse phase column (4.6 × 250 mm, Proteonavi C4, OSAKA SODA Co., LTD.) with 0.1% aq. TFA and 0.1% TFA in MeCN as solvent A and B respectively. 10 min of isocratic elution at a solvent A / B ratio of 99 / 1 was followed by a 40 min linear gradient to 100% solvent B at room temperature with a flow rate of 1 mL / min. Sample elution was detected using a UV detector at 254 nm.

2.4 Experimental animals

Fertilised Boris Brown eggs were purchased from Nihon Layer Co. Ltd (Gifu, Japan). Eggs were incubated at 38.0 °C in a humidified rocking egg incubator (Showa Furanki, Saitama, Japan), for approximately ten days until the embryos reached Hamburger and Hamilton Stage 36 (Hamburger and Hamilton, 1951). All experimental protocols were reviewed and approved in advance by either RIKEN Wako Animal Experiments Committee or Kyoto University Animal Experimentation Committee.

2.5 Dissociated neuron culture

On reaching the appropriate developmental stage, embryos were removed from their eggs and decapitated. Thoracic dorsal root ganglia were dissected from the embryos in cold phosphate-buffered saline (PBS; Thermo Fisher Scientific, DC), and the ganglia were collected in cold L15 medium (Thermo Fisher Scientific, DC). The neurons were then dissociated by repeated trituration, and plated at a density of approximately 10,000 cells per dish on glass base dishes that had been pre-treated with poly-D-lysine (Sigma-Aldrich, MO) and 12 µg/mL laminin (Thermo Fisher Scientific, DC). The dissociated neurons were cultured in either Dulbecco's Modified Eagle's Medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Life Technologies, CA) and 1% penicillin-streptomycin (Sigma-Aldrich, MO), or L15 medium supplemented with 1% BSA (Life Technologies, CA), 1% N2 Supplement (Thermo Fisher Scientific, DC), and 0.02% NGF (Sigma-Aldrich, CA). NGF was added to enrich the cultures for NGF-dependent, TrkA-expressing nociceptive neurons, as previously described (Guy *et al*., 2015).

2.6 *In vitro* axon turning assays

Axon turning assays were carried out using an IX81 inverted microscope (Evident Corporation, Tokyo, Japan) inside a humidified chamber maintained at 37 °C, as previously described (Guy *et al*., 2015). The growth cone of a single neuronal axon was exposed to a microscopic concentration gradient of lipid compound for a duration of 45 minutes. The growth cone was monitored continuously and images captured using a CCD digital camera (QImaging, Burnaby, Canada) controlled by AquaCosmos software (Version 2.6, Hamamatsu Photonics, Hamamatsu, Japan). The microscopic concentration gradient was generated within the culture dish by pulsatile ejection of lipid solution from a glass micropipette using positive nitrogen gas pressure, as previously described (Lohof et al., 1992). The pulsatile ejection frequency was 2 Hz and the pulse duration was 20 ms, regulated by an AWG-50 pulse generator (ELMOS, Osaka, Japan) linked to a PV820 Pneumatic PicoPump (World Precision Instruments, FL). Micropipettes were made from glass capillaries (borosilicate glass with filament, catalog number BF100-50-10; Sutter Instrument, CA), pulled on a P-97 Flaming/Brown micropipette puller (Sutter Instruments, CA) to have a steep shoulder and a tip diameter of approximately 1-2 µm, and each micropipette was visually checked on a microforge (Narishige, Tokyo, Japan). A healthy, straight-growing axonal growth cone was selected, imaged for stability for 10 minutes before starting, and positioned 100 µm from the tip of the micropipette at an angle of 45^{o} relative to the direction of growth of the axon. EtherLysoPtdGlc was dissolved in a vehicle of 0.1% (v/v) DMSO/PBS to a concentration of either 10 µM or 20 µM ("double etherLysoPtdGlc") and backloaded into the micropipette immediately prior to the turning assay. Where used in the turning assay, NGF was diluted in PBS to a final concentration of 50 µg/mL; Semaphorin 3A (R&D Systems, MN) was diluted in PBS to a concentration of 10 µg/mL; and lysophosphatidylinositol (LysoPtdIns; #850091P, Avanti Polar Lipids, AL) was diluted in PBS to 20 µM before being backloaded into the micropipette. Synthetic compounds 1a, 1b or 2 were dissolved in DMSO to make a

stock solution of 10 mM or 20 mM and stored in aliquots at -30°C until required. Before using in the biological assay, the stock was diluted in culture medium to give a final working concentration of 5 μM in the cell culture dish, to be used as either a bath application or administered as a pulse. For bath application 1a, 1b or 2, or the equivalent amount of DMSO as a control, was added to the culture medium 40 minutes before the start of the turning assay, and the medium was not changed for the duration of the experiment. For pulse treatment, the culture medium containing 1a, 1b, 2 or DMSO was added to the dissociated neuron cultures, and the dishes were placed in a 5% CO_2 incubator at 37.0 °C. After 4 or 8 hours pulse, the medium containing 1a, 1b, 2 or DMSO was removed from the culture dishes by suction, and the dishes further washed gently with medium three times. The cells were placed back in the incubator overnight in DMEM medium, for an average of 13 hours, and changed back to L15 medium before commencing the turning assay. At the commencement of the assay, pulsatile positive gas pressure was applied to the lipid solution in the micropipette, and the growth cone and axon imaged at the start and after 45 minutes. After 45 minutes exposure to the microscopic concentration gradient of etherLysoPtdGlc or control guidance cue, the axon turning angle was measured by comparing the initial direction of growth to the final direction of the growth cone, using ImageJ (National Institutes of Health, MD) software. The distance between the centre of the growth cone at the start and the end of the assay was measured in microns, using ImageJ, as a measure of axon health. Growth cones that divided, collapsed, retracted, or grew less than 10 μm, during the assay were excluded. All turning assays were conducted with the experimenter blind to the identity of the lipid or compound inside the micropipette.

2.7 Density function theory (DFT) calculations

DFT calculations were performed utilising the Gaussian 16 software on the RIKEN internal HOKUSAI cluster.

2.7.1 HOMO-LUMO calculations

The energy landscape for all model compounds was scanned with 60 degree increments of the freely rotatable bonds and the conformation with lowest energy was minimised without constraint using the B3LYP/aug-cc-pVTZ basis set and water as implicit solvent. HOMO and LUMO orbital energies were extracted at the respective optimised geometries and visualised using the Gaussview 6.0.16 software package at an isosurface value of 0.04.

2.7.2 Conformational energy landscape and degree of similarity

All geometries were optimised at the MP2/6-31+g(d) level followed by single point energy calculations. The dihedral angle of the anomeric linkage is referred to as ρ, measured between O(ring)-C(ring)-O(ester)-P for TPMe, O(ring)-C(ring)-N-C(squarate vinyl C) for type 1 and O(ring)-C(ring)-S-C(squarate vinyl C) for type 2 models respectively. The second dihedral angle is referred to as ψ and is measured between C(ring)-O(ester)-P-O(ester) in TPMe, C(ring)-N-C(squarate vinyl C)-C(squarate vinyl C) for type 1 and C(ring)-S-C(squarate vinyl C)-C(squarate vinyl C) for type 2 models respectively. Potential energy scans of TPMe were used *gauche+*, *gauche–* and *anti* orientation of the phosphate methyl ester as starting orientation and the lowest energy conformation was selected to generate the plot in figure S2A. Potential energy scans of type 1 and type 2 models used *syn* and *anti* orientation of the methyl moiety to the bridging squarate moiety as starting orientation and the lowest energy conformation was selected to generate the plots in figure 2A and 2D. All 2D plots were prepared utilising the GNUplot 4.6 software package.

The lowest energy conformation of TPMe was screened against all conformations of type 1 and 2 models, by superposition of their respective tetrahydropyrane moieties. The degree of similarity plots (Fig. 2C and 2E) were calculated as the sum of the distance between the respective terminal methyl groups carbon atoms and between the closest carbonyl oxygen of the squarate moiety to the non-bonding oxygen of the phosphate moiety in TPMe. Superposition and visualisation of the superimposed structures was performed using the VMD [Humphrey, A. Dalke, K. Schulten; J. Molec. Graphics, 1996, 14, 33-38] software package.

2.8 Docking analysis

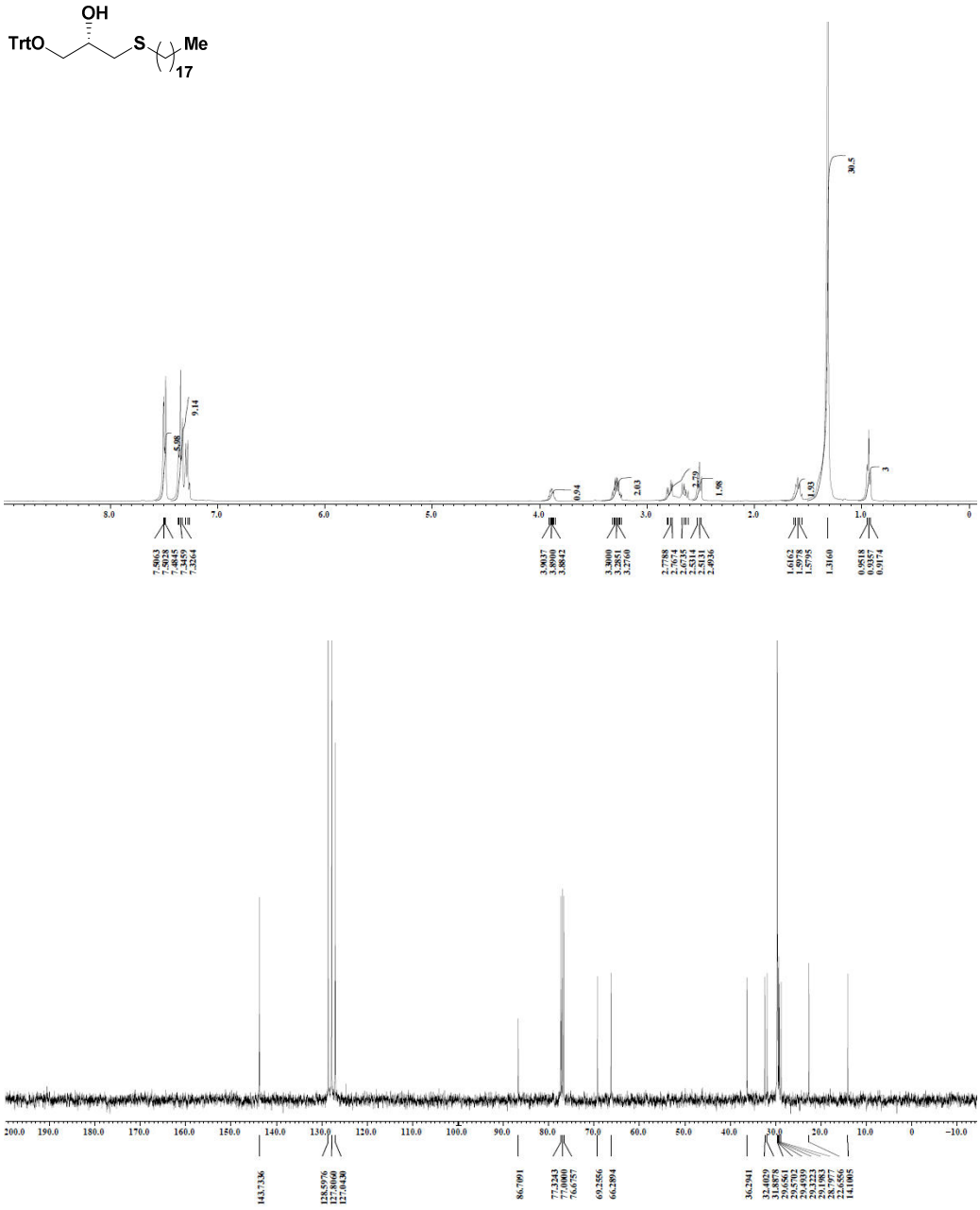
The PDB file for GPR55 was extracted from our previously reported[13] active state homology model of GPR55 following 50ns equilibration in a membrane environment in the presence of LysoPtdGlc, excluding all lipid

and molecules as well as ions. Residue names and atom names were adjusted as required and PDB files for compounds **1a** and **2** were extracted from DFT calculations at the B3LYP/6-31g(d) level. Flexible docking calculations were performed using AutoGrid4 (version 4.2.6) and AutoDock4 (version 4.2.6), employing standard protocol procedures. The resulting clusters were ranked by their predicted binding energies and the distance between $K80^{2.60}$ N_{ϵ} and the squarate vinyl carbon (CV) adjacent to the alkylthiolate was evaluated. The five conformation clusters with the lowest binding energy exhibited an average lysine Nε to CV distance of 0.44±0.05 nm and 0.40±0.06 nm for compound **1a** and **2** respectively.

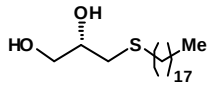
3. Supplementary Data

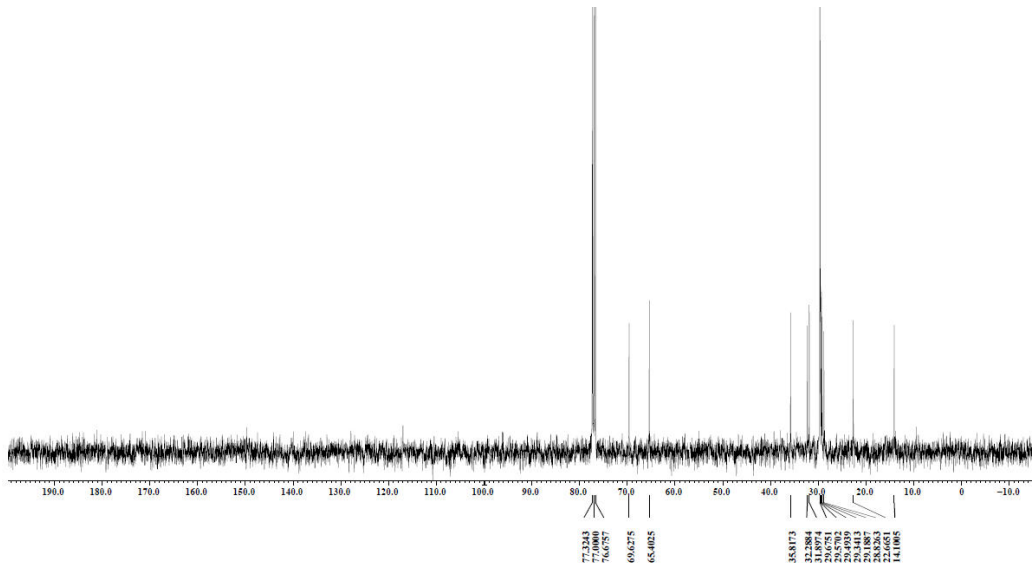
3.1 Compound characterisation by ^{1}H and ^{13}C NMR

3.1.1 (*S*)-1-(octadecylthio)-3-(trityloxy)propan-2-ol (4)

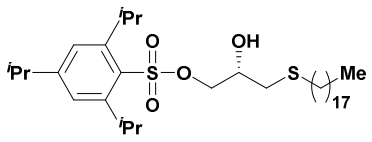


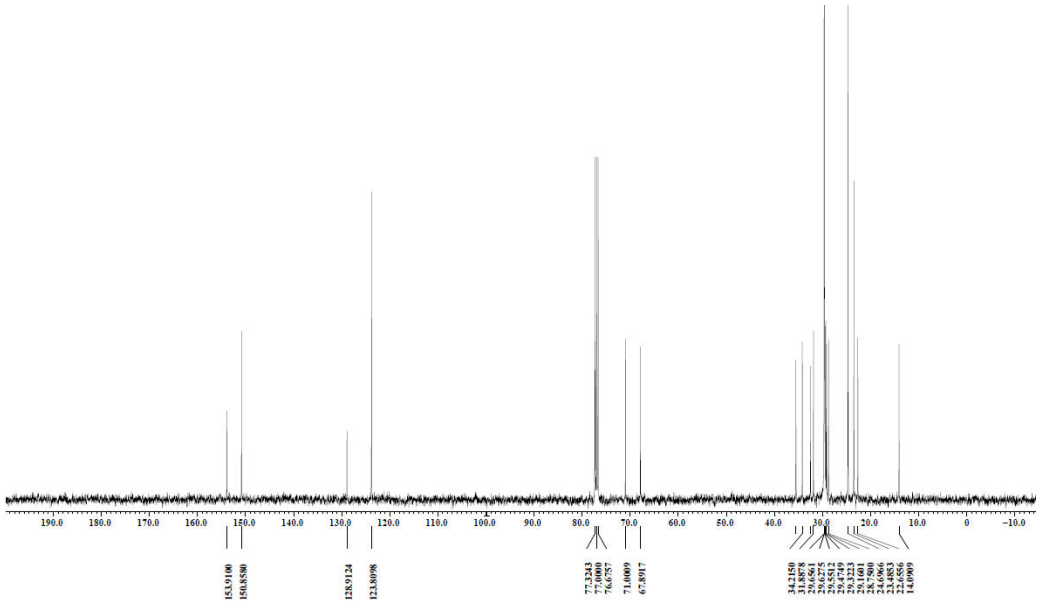
3.1.2 (*S*)-3-(octadecylthio)propane-1,2-diol (5)



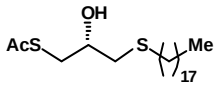


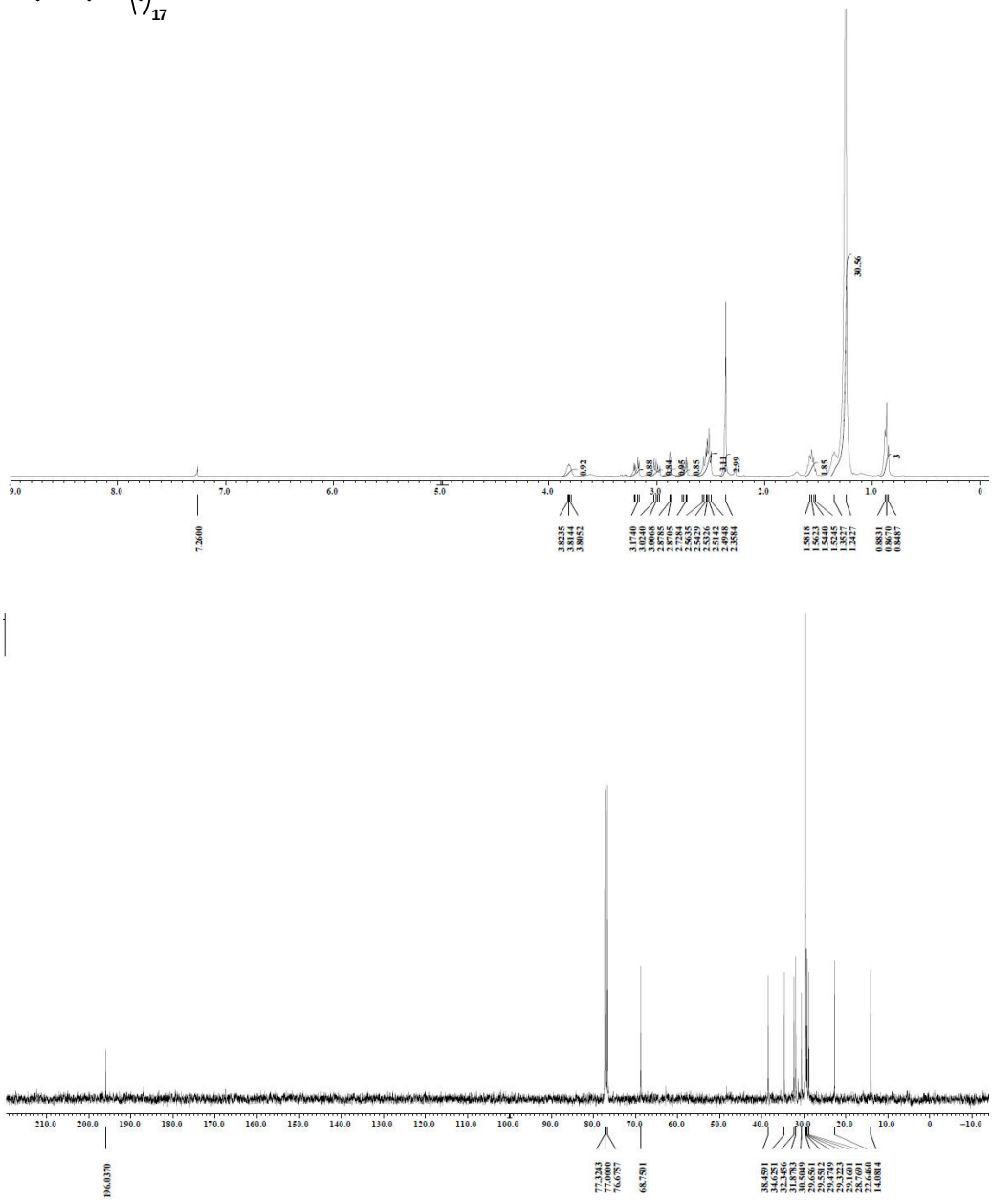
3.1.3 (*S*)-2-hydroxy-3-(octadecylthio)propyl 2,4,6-trimethylbenzenesulfonate (6)



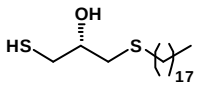


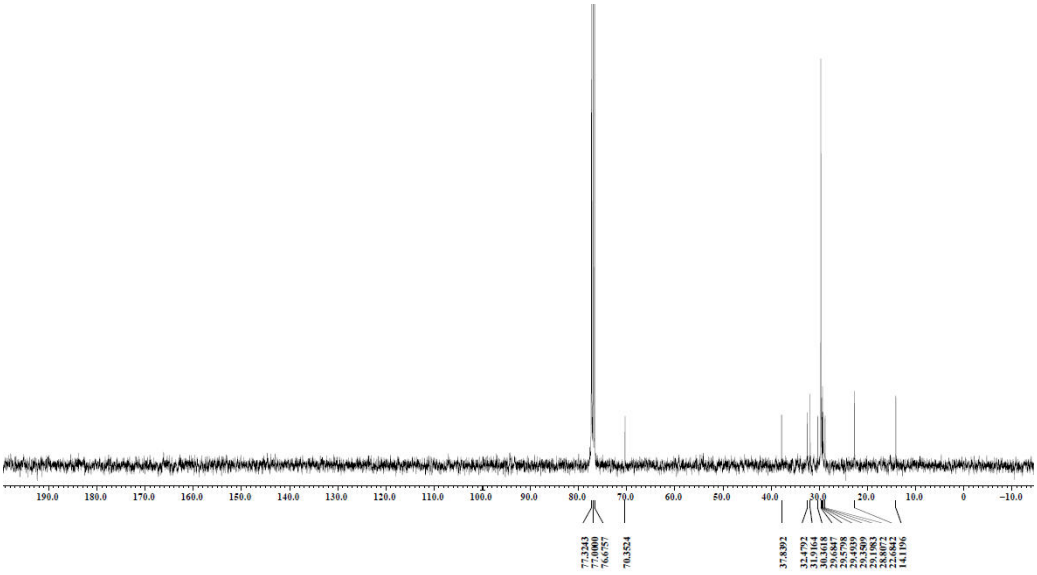
3.1.4 (*R*)-S-(2-hydroxy-3-(octadecylthio)propyl) ethanethioate (7)



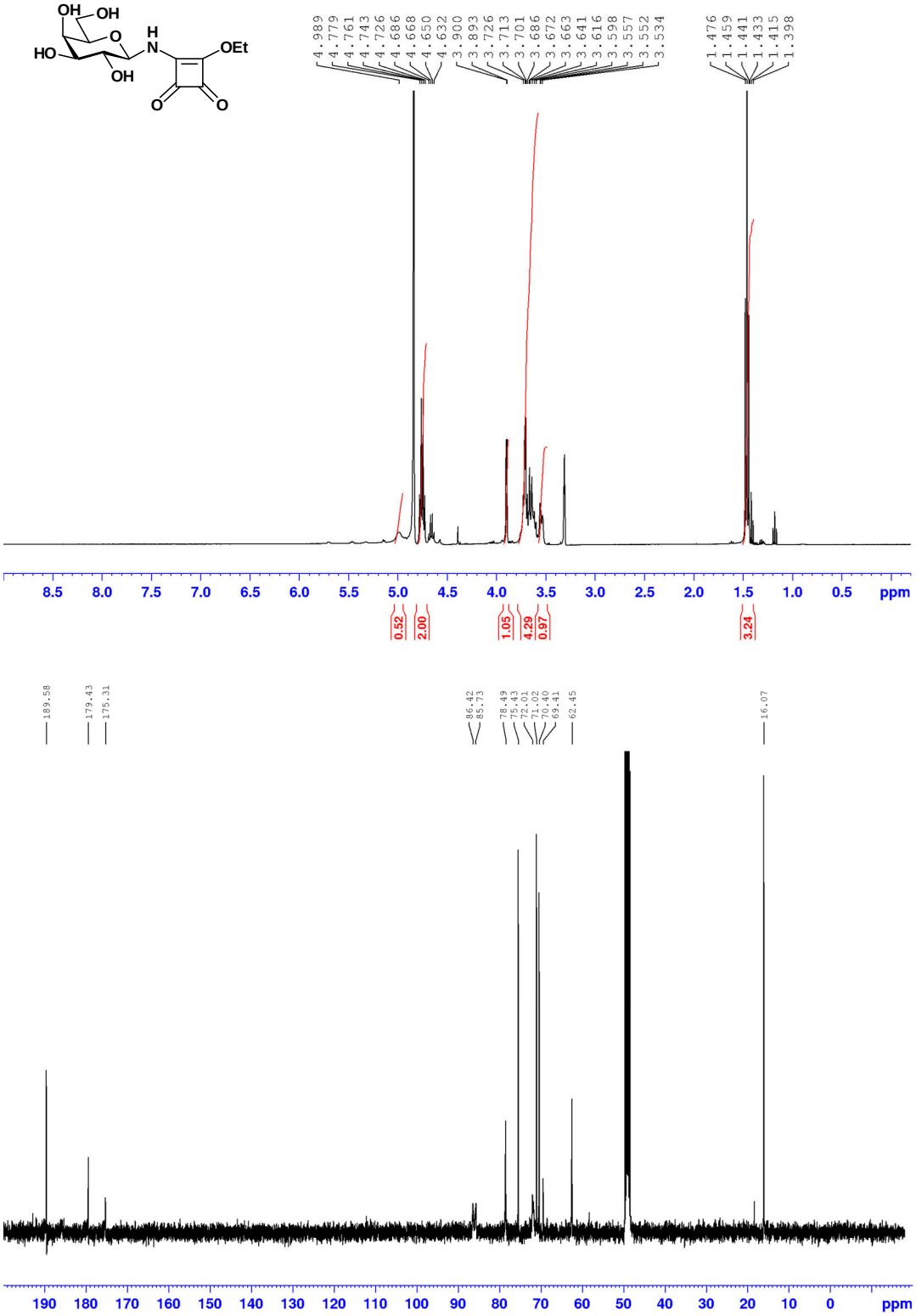


3.1.5 (*S*)-1-mercapto-3-(octadecylthio)propan-2-ol (8)

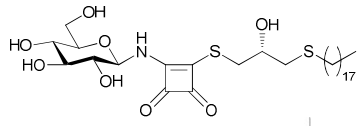


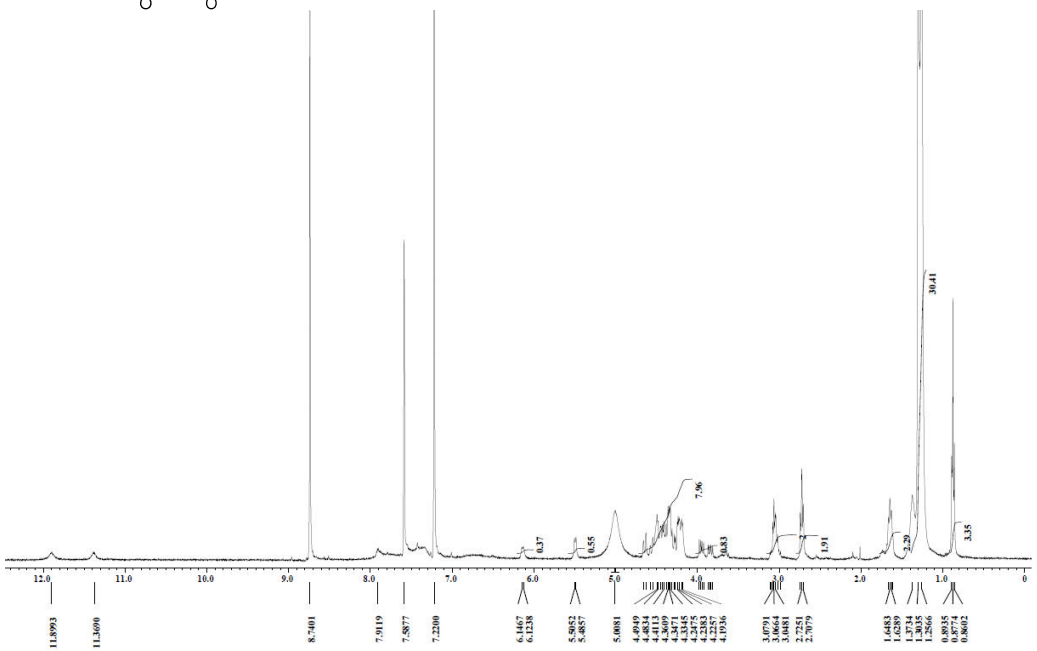


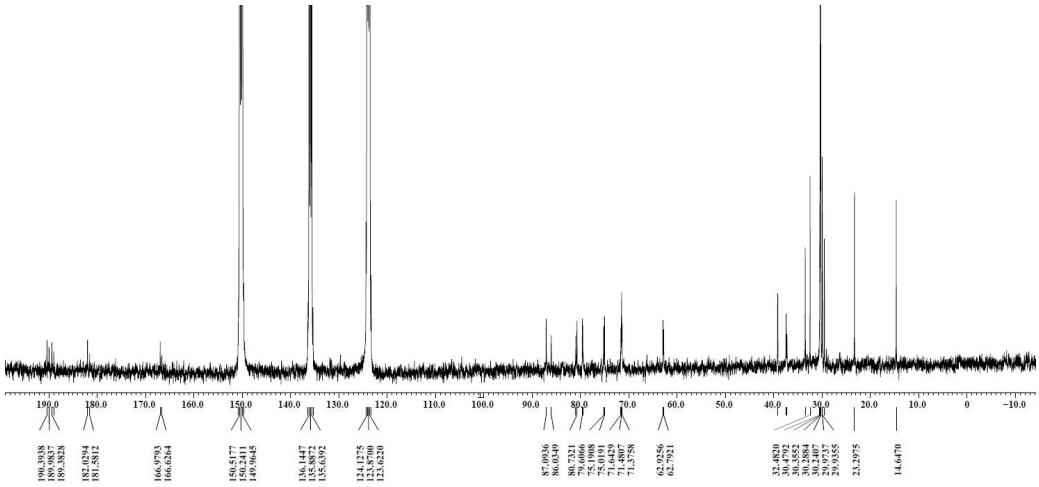
3.1.6 3-ethoxy-4-(*N*-β-D-galactopyranosylamino)cyclobut-3-ene-1,2dione (9b)



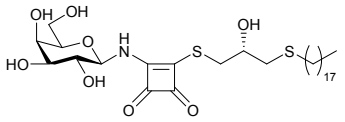
3.1.7 3-(((*R*)-2-hydroxy-3-(octadecylthio)propyl)thio)-4-(*N*-β-D-glucopyranosylamino)cyclobut-3-ene-1,2dione (1a)

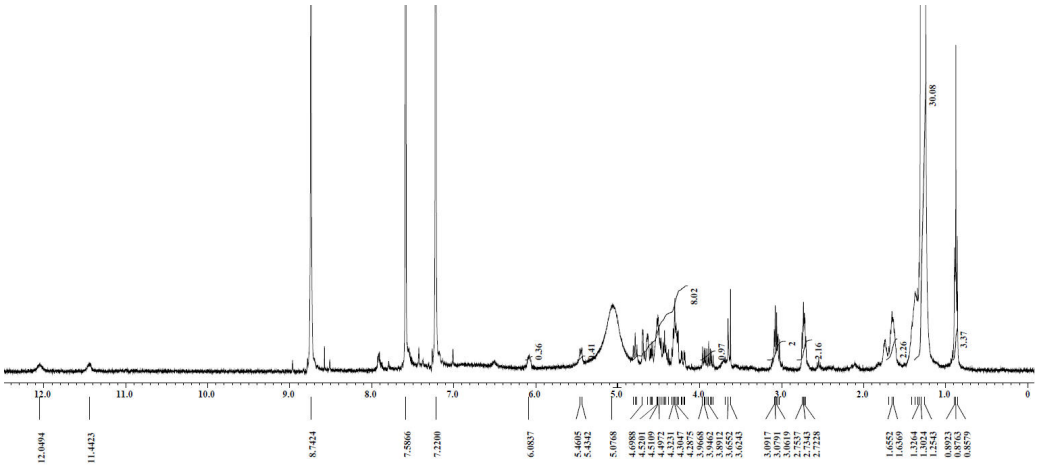


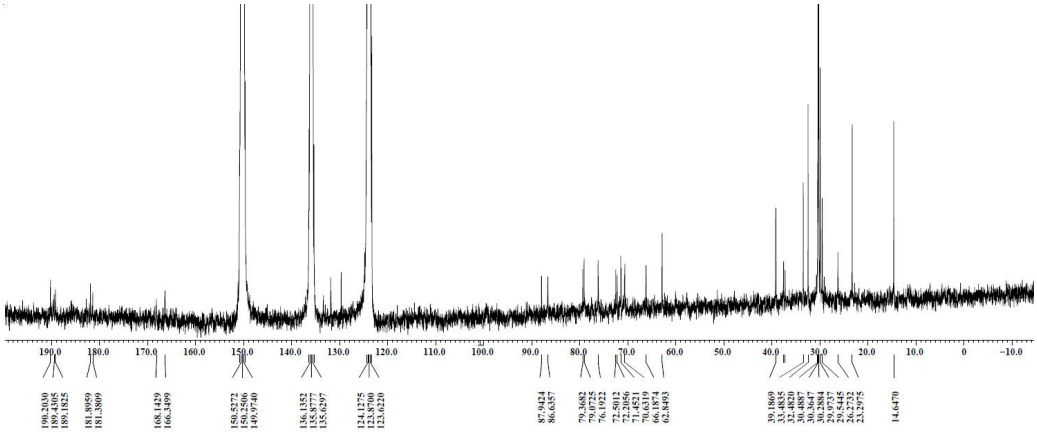




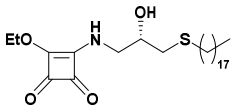
3.1.8 3-(((*R*)-2-hydroxy-3-(octadecylthio)propyl)thio)-4-(*N*-β-D-galactopyranosylamino)cyclobut-3-ene-1,2dione (1b)

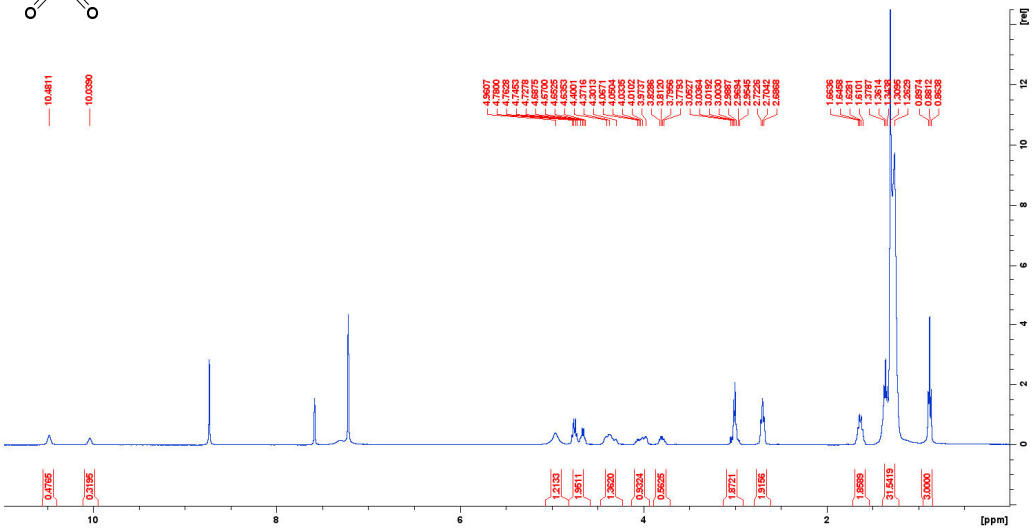


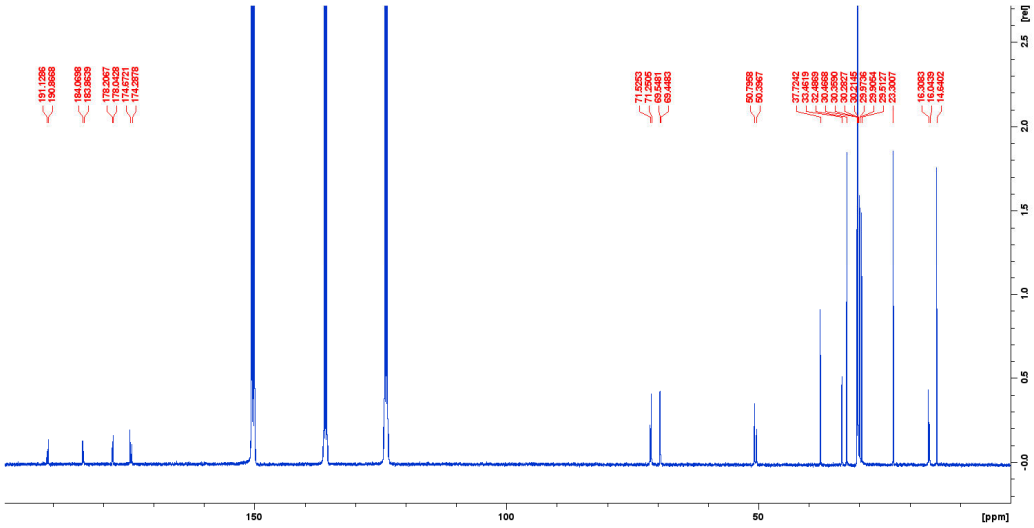




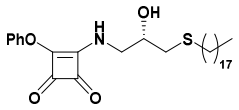
3.1.9 (*S*)-3-ethoxy-4-((2-hydroxy-3-(octadecylthio)propyl)amino)cyclobut-3-ene-1,2dione (11a)

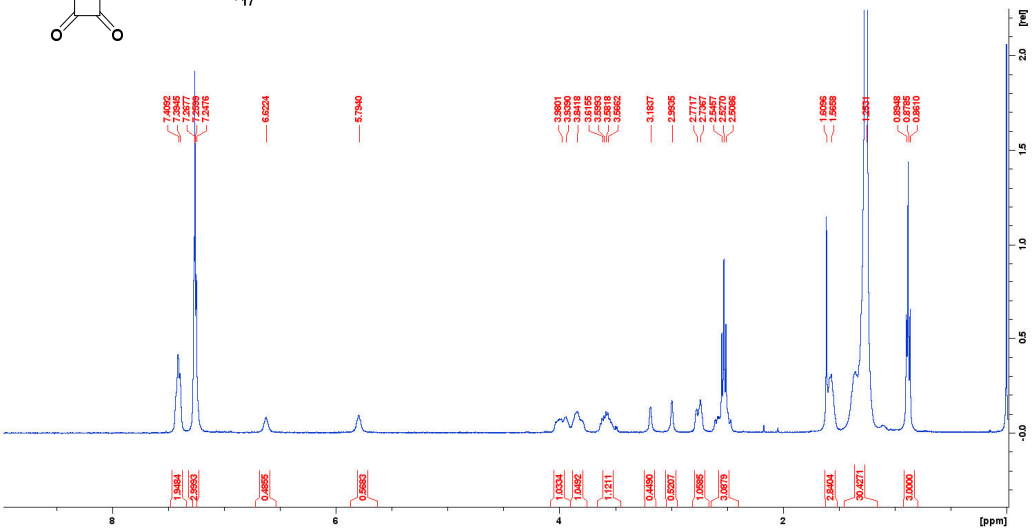


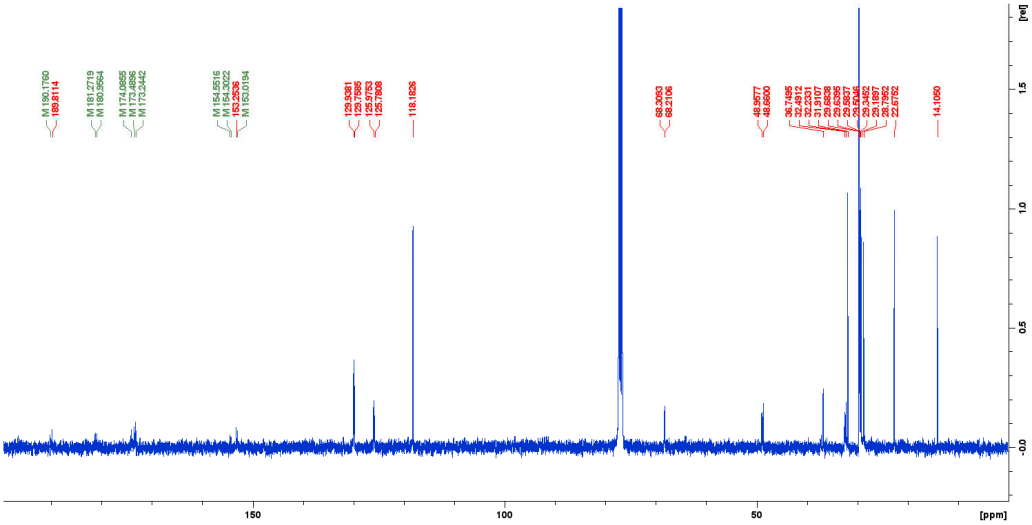




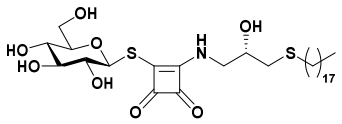
3.1.10 (*S*)-3-((2-hydroxy-3-(octadecylthio)propyl)amino)-4-phenoxycyclobut-3-ene-1,2dione (11b)

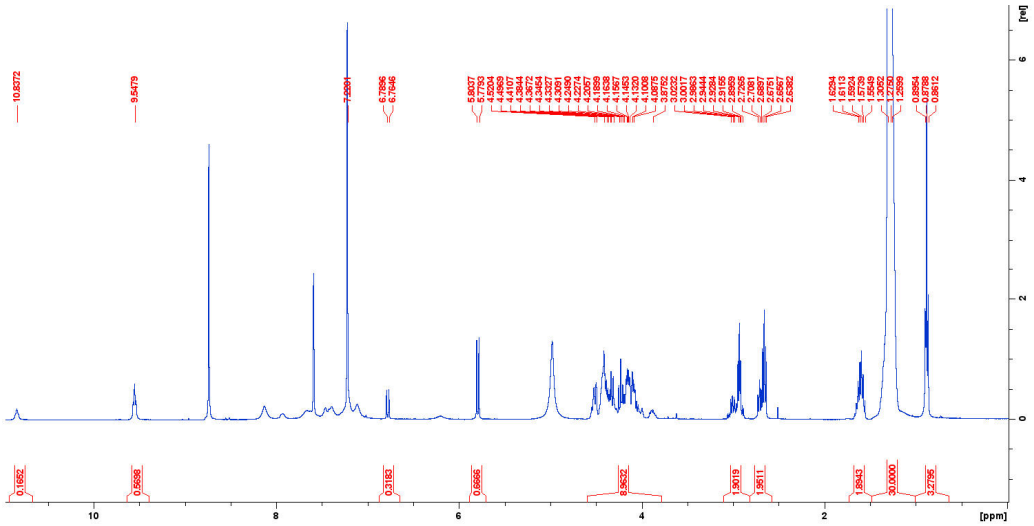


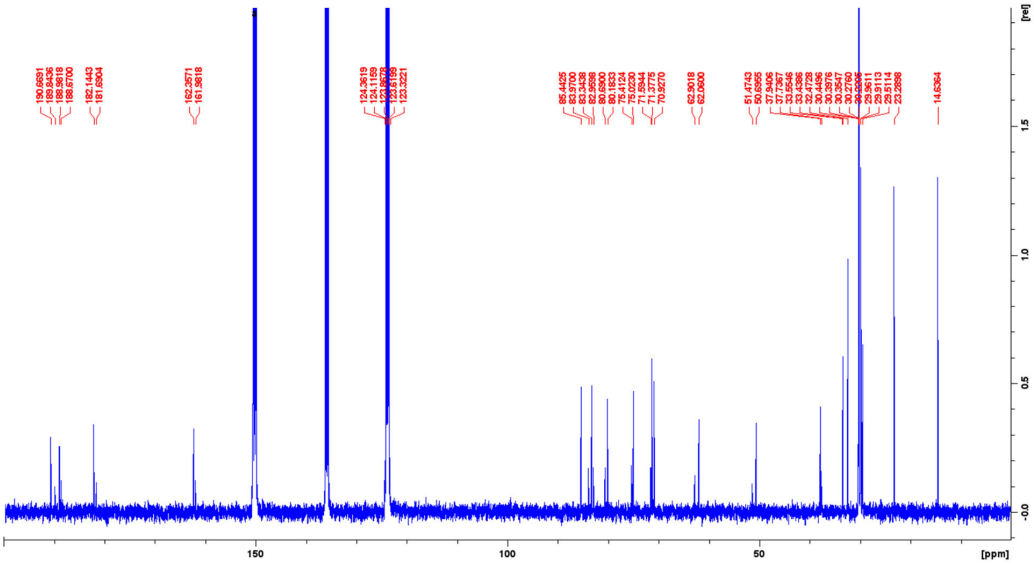




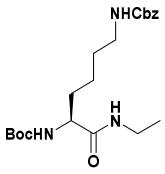
3.1.11 3-(((*S*)-2-hydroxy-3-(octadecylthio)propyl)thio)-4-(*S*-β-D-galactopyranosylthio)cyclobut-3-ene-1,2dione (2)

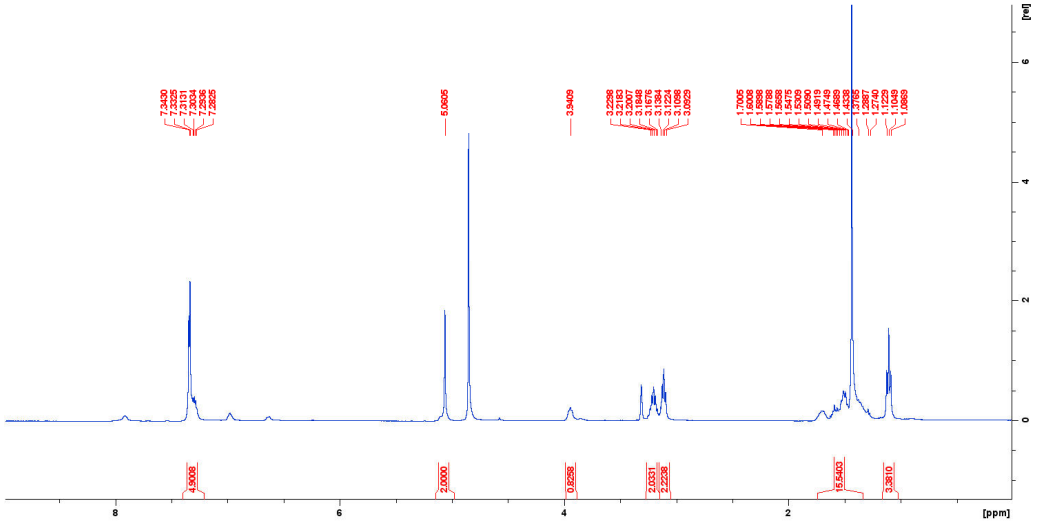


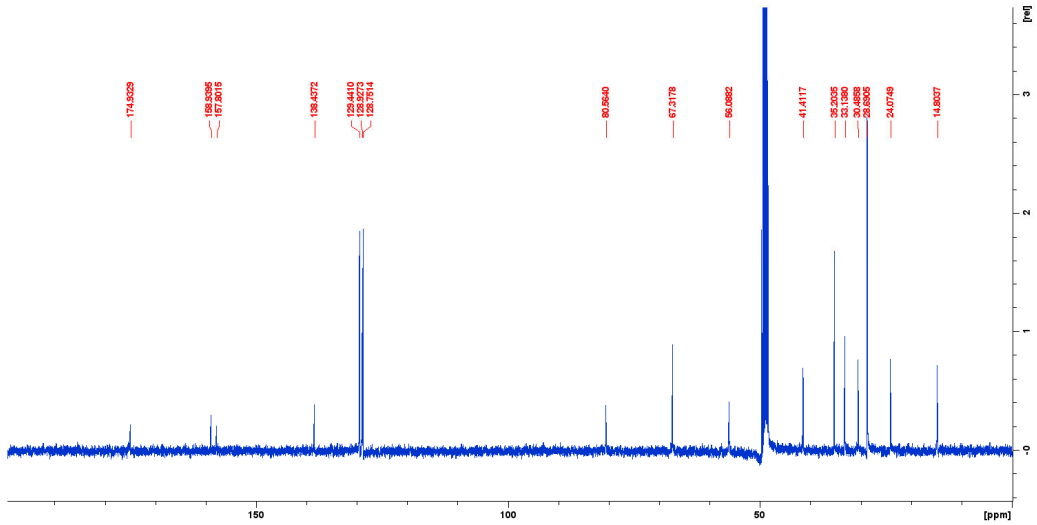




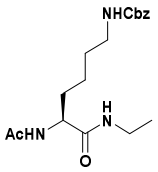
3.1.12 (*S*)-benzyl *tert*-butyl (6-(ethylamino)-6-oxohexane-1,5-diyl)dicarbamate (14)

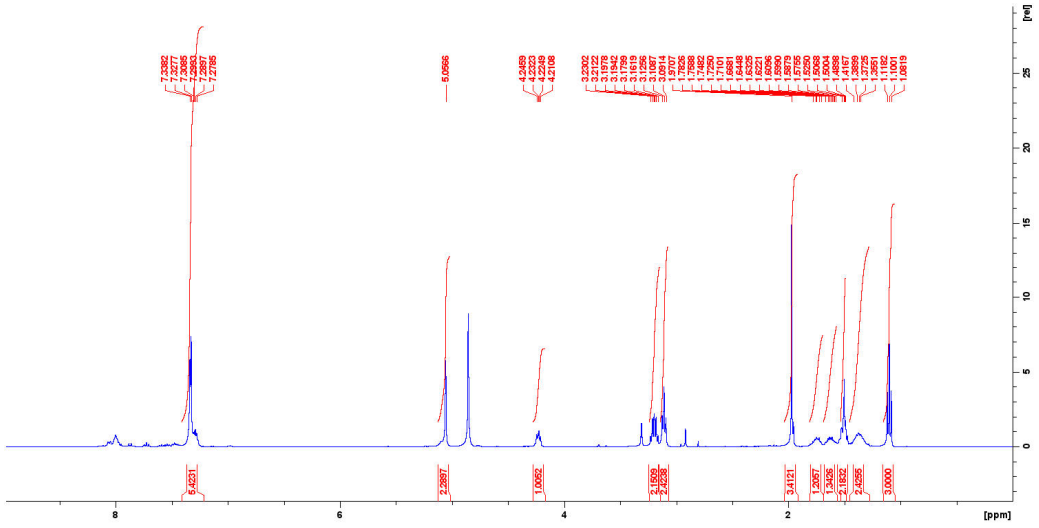


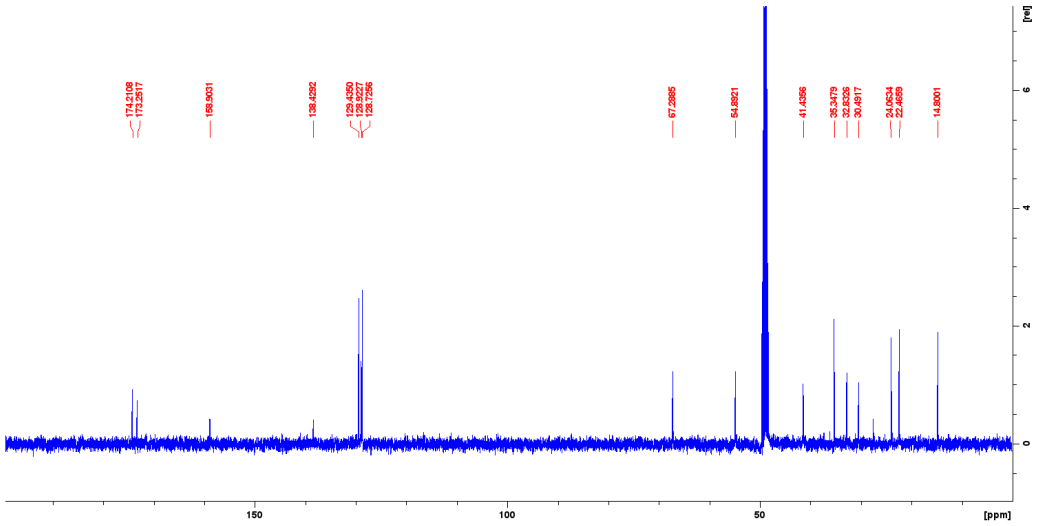




3.1.13 (*S*)-benzyl (5-acetamido-6-(ethylamino)-6-oxohexyl)carbamate (15)







3.1.14 (*S*)-2-acetamido-6-amino-*N*-ethylhexanamide (13)