Replacing D-glucosamine with its L-enantiomer in glycosylated antitumor ether lipids (GAELs) retains cytotoxic effects against epithelial cancer cells and cancer stem cells

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Supplementary Data

Table S1. CC_{50} and CC_{90} values of **1 – 5,** adriamycin, cisplatin and salinomycin against epithelial cells from human breast cancer (JIMT-1, MDAMB-231, BT-474), human prostate cancer (PC-3, DU-145) and human pancreas cancer (MiaPaCa-2).[c]

	JIMT-1		MDAMB231		MiaPaCa-2		PC-3		BT-474		DU-145	
	CC_{50} (µM)	CC_{90} (µM)	CC_{50} (µM)	CC_{90} (µM)	CC_{50} (µM)	CC_{90} (µM)	CC_{50} (µM)	CC_{90} (µM)	CC_{50} (µM)	CC_{90} (µM)	CC_{50} (µM)	CC_{90} (µM)
1	6.0	9.5	ND	ND	7.0	13.0	10.5	20.0	6.2	18.0	7.0	9.5
2[a]	6.5	11.0	11.0	17.0	7.5	18.0	12.5	16.0	13.0	18.0	12.5	16.0
3	4.0	6.5	5.5	7.5	6.0	15.0	6.5	9.0	11.0	14.0	6.0	8.0
4	2.0	3.5	4.0	4.9	5.0	7.1	2.6	4.3	6.0	7.3	3.6	4.9
5	2.0	3.5	4.0	4.9	4.5	7.0	3.5	4.6	6.0	7.3	3.6	4.9
Adriamycin	ND	ND	0.08	>2.0	0.3	>2.0	ND	ND	0.6	>2.0	1.3	>2.0
Cisplatin	ND	ND	>20	>20	>20	>20	ND	ND	>20	>20	14.8	>20.0
Salinomyin	ND	ND	>20	>20	6.5	>20	ND	ND	14.0	>20	>20	>20.0

[c]The CC_{50} and CC_{90} values are defined as the concentrations required to decrease cell viability by 50% and 90% respectively, relative to the untreated control. The values were obtained by estimating the drug concentration at 50% and 10% viability on the y-axis using line plots. ND = Not determined. [a]Compound **2** contained traces < 5 % of the corresponding β-anomer.

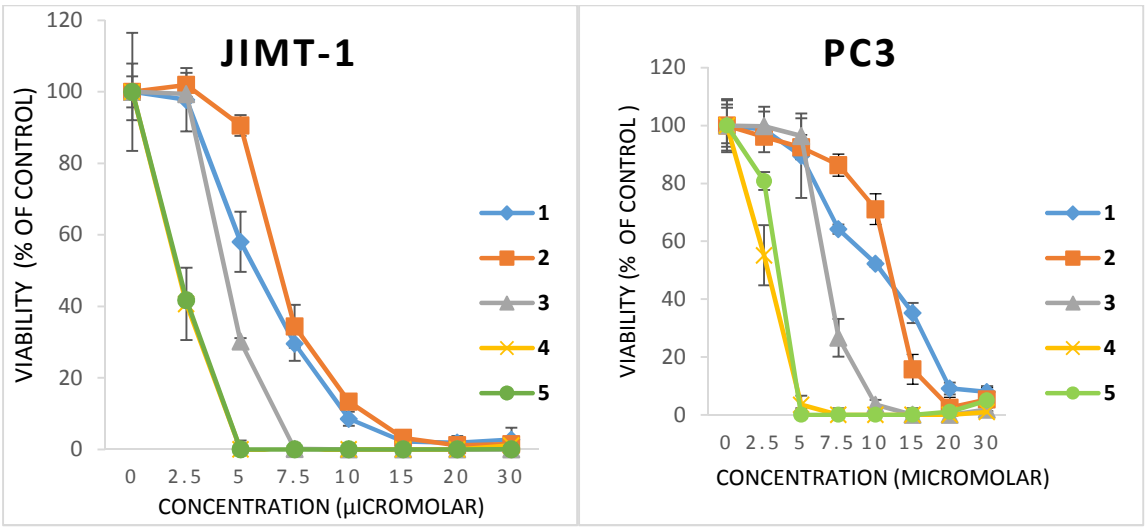


Figure S1: Effects of compounds **1-5**, cisplatin and salinomycin on the viability of PC-3 and JIMT-1 cell lines assessed by using the MTS assay after 48 h. The results represent the mean ± standard deviation of 6 independent determinations.

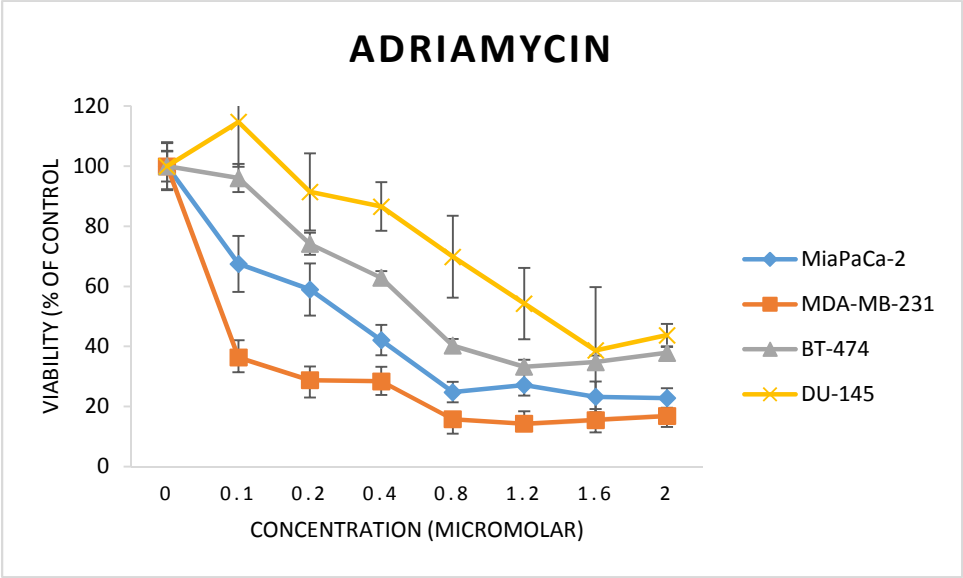


Figure S2: Effects of adriamycin on the viability of MiaPaCa-2, MDA-MB-231, BT-474 and DU-145 cell lines assessed by using the MTS assay after 48 h. The results represent the mean ± standard deviation of 6 independent determinations.

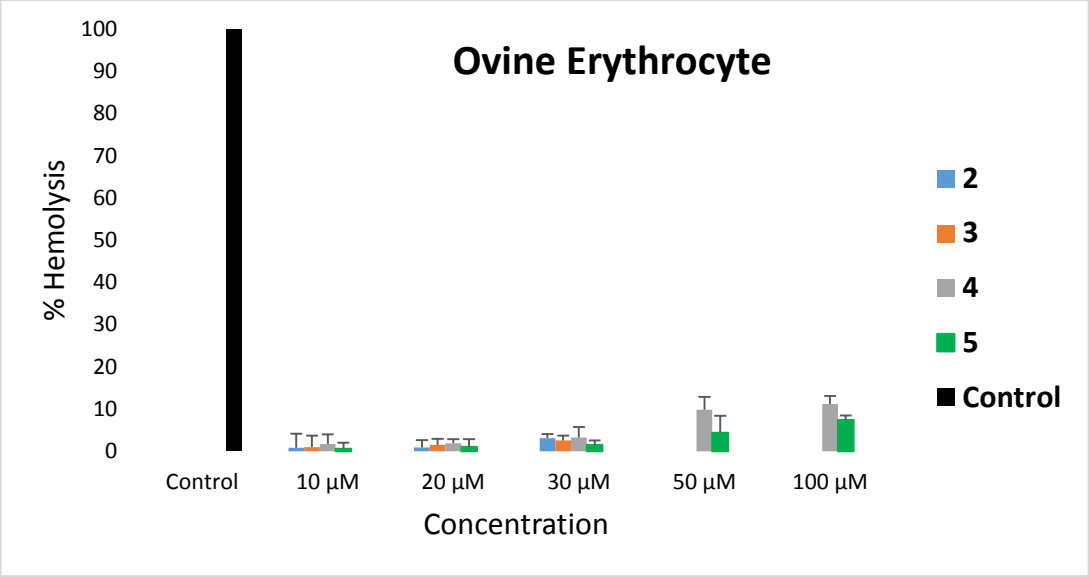


Figure S3: Hemolytic properties of GAELs **2 - 5** on ovine erythrocytes. The results represent the mean ± standard deviation of 4 independent determinations. The hemolysis was calculated as a percentage of the control, 1% NH_4OH, which achieved 100% hemolysis.

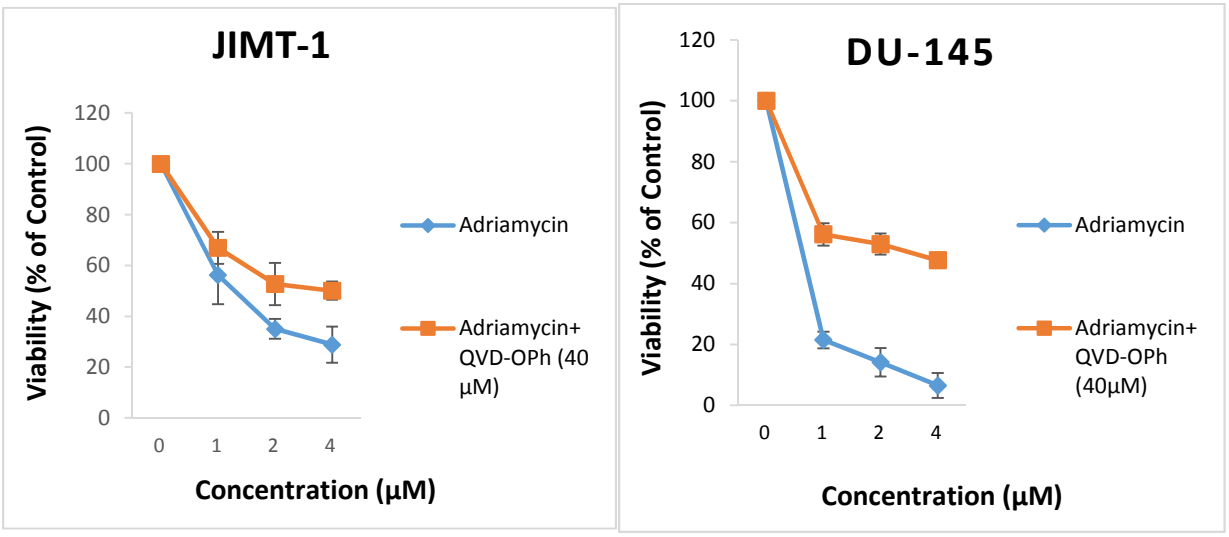


Figure S4: Effect of pan-caspase inhibitor, QVD-OPh (40 µm) on cytotoxicity of adriamycin against DU-145 and JIMT-1 cancer cell lines. Cancer cell lines were treated with drugs in the presence or absence of QVD-OPh before viability was assessed with MTS assay. The results represent the mean ± standard deviation of 6 independent determinations.

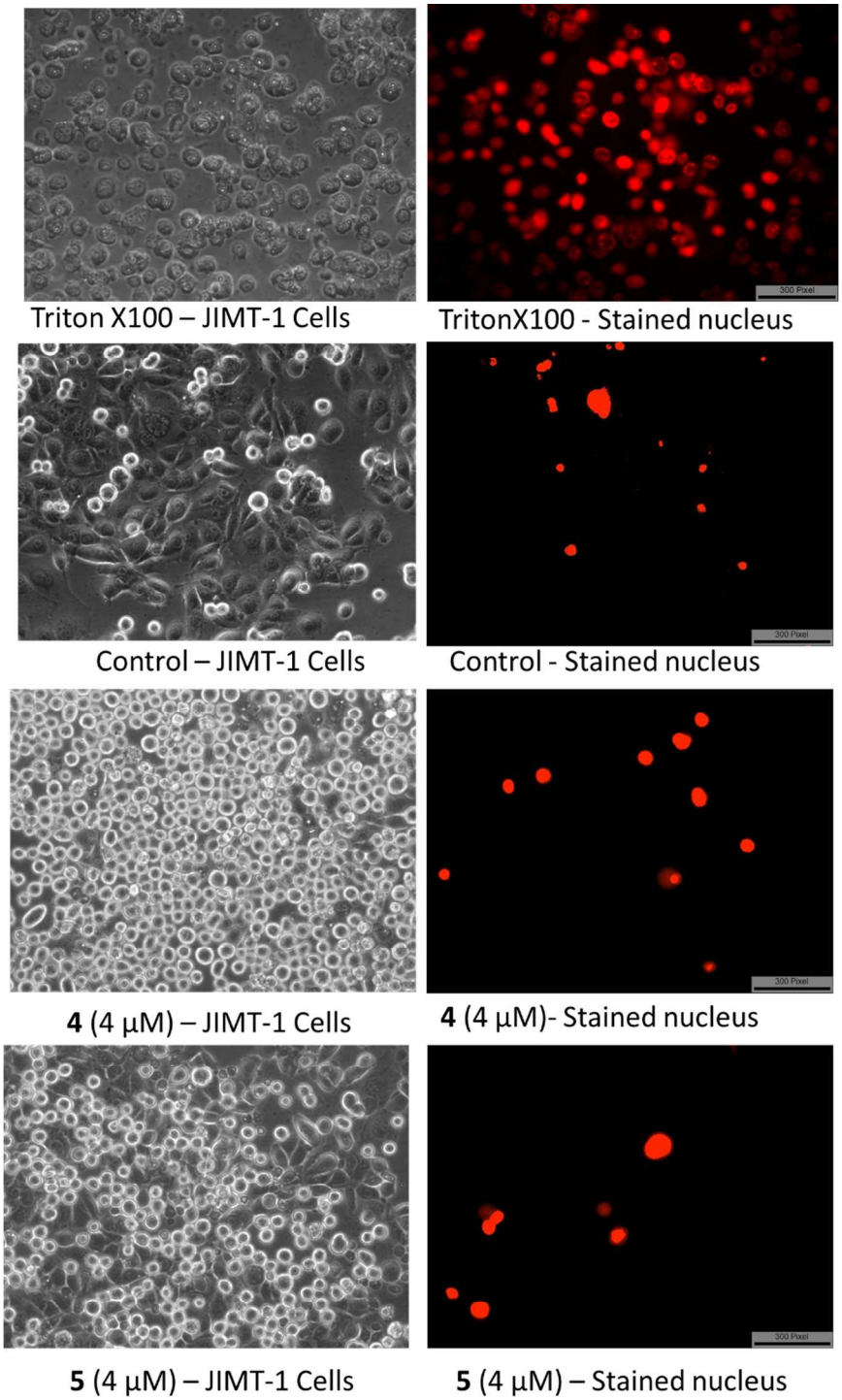


Figure S5: Evaluation of membranolytic effect of compounds **4** and **5** on JIMT-1 cell lines using cell impermeant ethidium homodimer-1 (EthD-1) dye that emits red fluorescence upon binding to DNA. The images were taken with an Olympus IX70 microscope at a magnification of x10.

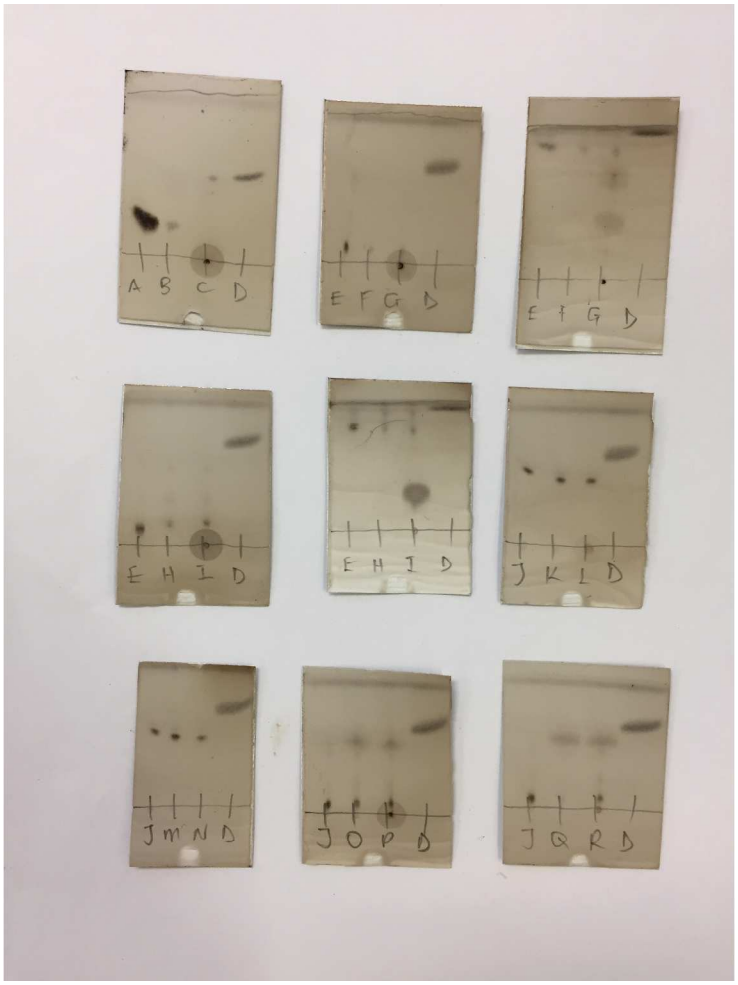


Figure S6: Thin-layer chromatographic analysis of the metabolic stability of D- and L-GAELs to bovine liver extracts and pure α-glucosidase enzyme purified from *Saccharomyces cerevisiae*.

A: pure β-D-glucose-based GAEL

B: β-D-glucose-based GAEL at pH 4.4 without liver extract

C: β-D-glucose-based GAEL at pH 4.4 with liver extract

D: pure glycerolipid

E: pure compound **2**

F: compound **2** at pH 4.4 without liver extract

G: compound **2** at pH 4.4 with liver extract

H: compound **2** at pH 6.8 without pure α-glucosidase enzyme

I: compound **2** at pH 6.8 with pure α-glucosidase enzyme

J: pure compound **4**

K: compound **4** at pH 4.4 without liver extract

L: compound **4** at pH 4.4 with liver extract

M: compound **4** at pH 6.8 without pure α-glucosidase enzyme

N: compound **4** at pH 6.8 with pure α-glucosidase enzyme

O: compound **2** solubilized in triton X-100 at pH 4.4 without liver extract

P: compound **2** solubilized in triton X-100 at pH 4.4 with liver extract

Q: compound **2** solubilized in triton X-100 at pH 6.8 without pure α-glucosidase enzyme

R: compound **2** solubilized in triton X-100 at pH 6.8 with pure α-glucosidase enzyme

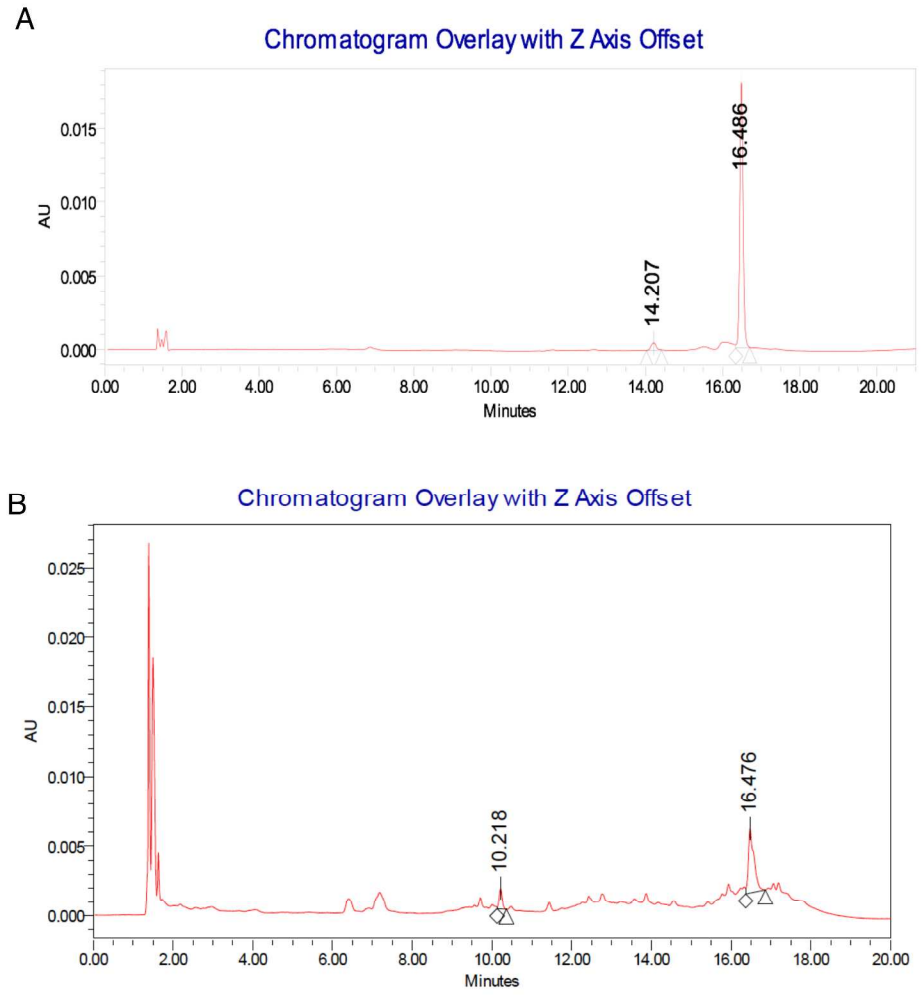
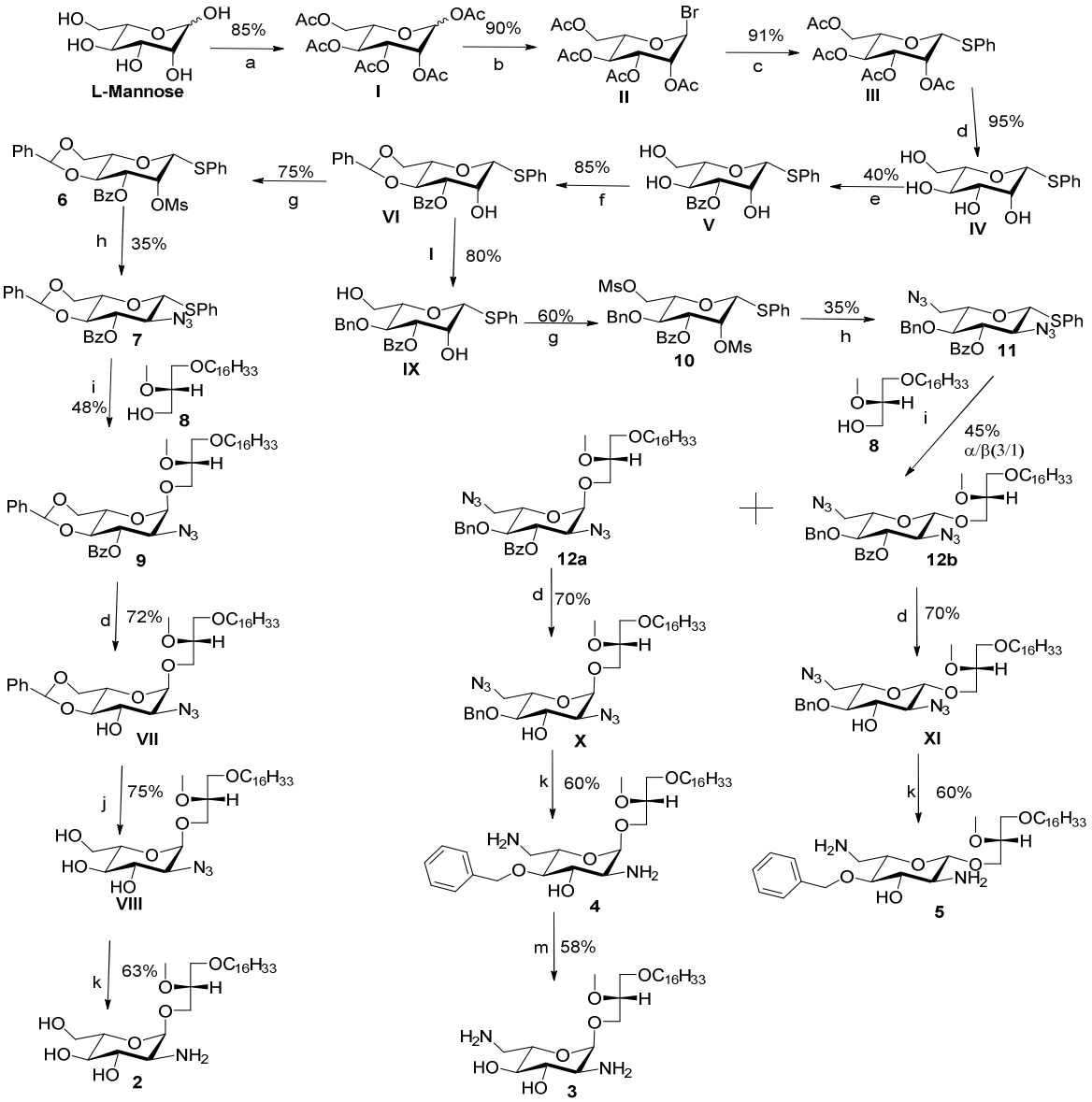


Figure S7: HPLC chromatogram of compound **4** (A) before and (B) after exposure to bovine liver extract for 4 h



Scheme S1. Synthesis of L-GAELs **2-5**. Reactions and Conditions; (a) Ac_2O, DMAP, pyridine, 18 h, rt (b) HBr in AcOH (33%), DCM, 0 °C, 2 h (c) PhSH, EtOAc, H_2O, Na_2CO_3, TBAHS, rt, 6 h (d) NaOMe, MeOH, 1h (e) Me_2SnCl_2, BzCl, DIPEA, THF/H_2O (19:1), rt, 16 h (f) $PhCH(OMe)_2$, CSA, CH_3CN, rt, 4 h (g) MsCl, pyridine, DMAP, rt, 18 h (h) DMF, NaN_3, 120 °C (i) AgOTf, NIS, DCM, 3 h, rt (j) AcOH, H_2O, 60 °C, 5 h (k) $P(CH_3)_3$, THF, H_2O, 2 h, rt (l) BH_3.THF, TMSOTf, DCM, 4 h (m) H_2, Pd/C, MeOH, 5 h

10. Experimental section

10.1. Chemistry

10.1.1. General information

Solvents were dried over CaH_2. ^1H, ^{13}C NMR spectra were recorded on a Bruker Avance NMR spectrometer (300 and 500 MHz), and chemical shifts reported (in ppm) relative to the indicated solvents. Thin-layer chromatography (TLC) was carried out on aluminum or glass-backed silica gel GF plates (250 mm thickness), and visualized by charring with 5% H_2SO_4 in methanol and/or short wavelength UV light. Compounds were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh). Mass spectrometric analyses were carried out on ESI Varian 500 MS Ion Trap Mass Spectrometer and MALDI-TOF Bruker Daltonics Ultraflex Spectrometer. Purity of compound **2** was assessed by elemental analysis of constituent elements (C, H, N), and were found to be within ± 0.5 % of the theoretical values. Purity of compounds **3 – 5** were assessed by HPLC-UV using a mobile phase gradient A: Water 0.1% TFA (Trifluoroacetic acid); B: Acetonitrile 0.1% TFA (Trifluoroacetic acid); Flow rate: 1 mL/min; Column: Kinetex 5 µm EVO RP C18 100 Å, 150 x 4.6 mm, Phenomenex Injection: 30 µL of 0.1 mg/mL; Equilibration time: at least 10 column volume of the initial gradient. The purity was > 95%. L-mannose and other chemicals used were purchased from Sigma-Aldrich Canada.

10.1.2. Synthesis of GAELs

1, 2, 3, 4, 6 – Penta-O-acetyl *α*/β *-L* mannopyranoside (I)

L-mannose (2 g, 11.10 mmol), was dissolved in pyridine (40 ml) and acetic anhydride (11 ml, 111 mmol), followed by the addition of a catalytic amount of DMAP (0.27 g 2.20 mmol). The mixture was stirred for 18 h at room temperature, stopped with methanol, and concentrated under high vaccum. The resulting residue was dispersed in ethyl acetate (50 ml), and washed with 3% HCl solution (×1), saturated sodium bicarbonate (×2), distilled water (×1) and brine (×1). The resulting organic layer was dried over Na_2SO_4, concentrated, and purified by flash chromatography using ethyl acetate and hexane (1:1) to give **I** (3.7 g, 9.48 mmol) as α:β mixture (4:1). Yield was 85%. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{16}H_{22}O_{11}Na^+$: 414.1, found: 414.5

2, 3, 4, 6-tetra-O-acetyl-*α-L* mannopyranosyl bromde (II)

Compound **I** (3.7 g, 9.48 mmol) was added at room temperature to 25 ml of HBr in AcOH (33% solution).[1] The reaction was stirred for 2 h and diluted with 25 ml of DCM. The resulting solution was transferred to a separatory funnel containing ice cold water and the organic layer washed with additional ice cold water until the pH became neutral. The organic layer was dried over anhydrous sodium sulphate and concentrated under vacuum to give α-anomer **II** (3.5 g, 8.54 mmol) as the desired product without further purification. Yield is 90 %. Compound **II** was stored at -20 °C until it was used. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.31 (d, *J* = 1.7 Hz, 1H, H-1), 5.73 (dd, *J* = 2.7,10.1 Hz, 1H, H-3), 5.46 (dd, *J* = 2.7, 1.2 Hz, 1H, H-2), 5.38 (dd, *J* = 10.1, 3.5 Hz, 1H, H-4), 4.34 (dd, *J* = 12.4, 4.9 Hz, 1H, H-6a), 4.29 – 4.19 (m, 1H, H-5), 4.15 (dd, *J* = 12.4, 2.2 Hz, 1H, H-6b), 2.18 (s, 3H), 2.11 (s, 3H), 2.08 (s, 3H), 2.02 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.50, 169.68, 169.56, 169. 53, 83.08, 72.87, 72.17, 67.95, 65.35, 61.47, 20.76, 20.68, 20.65, 20.57.[2] ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{14}H_{19}O_9BrNa^+$: 433.0, found: 432.9

Phenyl-2,3,4,6 tetra-O-acetyl-1-thio-*β-L*-mannopyranoside (III)

Compound **II** (1.0 g, 2.43 mmol) and thiophenol (0.36 ml, 3.60 mmol) were dissolved in 10 ml ethyl acetate, followed by the addition of 10 ml of 2 M sodium carbonate. The solution was stirred vigorously and TBAHS (0.82 g, 2.43 mmol) was added. The reaction mixture was stirred for 15 h at room temperature after which it was diluted with 40 ml of ethyl acetate and washed with saturated sodium bicarbonate (×2), distilled water (×1) and brine (×2). The organic layer was dried over anhydrous sodium sulphate, concentrated under vacuum, and purified by flash chromatography using ethyl acetate and hexane (2:3) to give **III** (0.97 g, 2.21 mmol) as off white solid. Yield is 91 %. Anomeric configuration was verified by $J_{H1\text{-}C1}$ =155.9 Hz.[3] ^{1}H NMR (300 MHz, MeOD) δ 7.66 – 7.19 (m, 5H, aromatic protons), 5.64 (d, *J* = 1.2 Hz, 1H, H_1), 5.37 – 5.15 (m, 3H), 4.89 (s, 2H), 4.29 (m, 1H), 4.18 – 4.07 (m, 1H), 3.90 (m, 1H), 2.19 (s, Hz, 3H), 2.08 (s, 3H), 2.05 (m, 3H), 1.97 (s, 3H). ^{13}C NMR (75 MHz, MeOD) δ 137.19, 132.50, 130.24, 85.77, 77.29, 73.16, 72.38, 67.12, 63.78, 20.82, 20.71, 20.60, 20.50. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{20}H_{24}O_9SNa^+$: 463.1, found: 463.4

Phenyl-1-thio-*β-L*-mannopyranoside (IV)

Compound **III** (0.97 g, 2.21 mmol) was suspended in 15 ml of methanol followed by the addition of catalytic amount of sodium methoxide (0.05 g). The solution was stirred for 1 h and the reaction stopped with acidic ion exchange resin (0.10 g). The resin was filtered and the filtrate was concentrated under vacuum. The residue was subsequently purified by flash chromatography using 100 % ethyl acetate to give **IV** (0.57 g, 2.10 mmol) as an off white solid. Yield is 95 %. ^{1}H NMR (300 MHz, MeOD) δ 7.57 – 7.18 (m, 5H, aromatic protons), 5.02 (d, *J* = 1.2 Hz, 1H, H-1), 4.08 (dd, *J* = 1.2, 3.3 Hz,1H, H-2), 3.90 (dd, *J* = 12.0, 2.4 Hz, 1H, H-6a), 3.77

(dd, J = 12.1, 5.6 Hz, 1H, H-6b), 3.67 (dd, J_1 = 9.6 Hz, J_2 = 9.6 Hz, 1H, H-4), 3.55 (dd, J = 9.5, 3.3 Hz, 1H, H-3), 3.39 – 3.25 (m, 1H). ^{13}C NMR (75 MHz, MeOD) δ 137.18, 131.02, 129.99, 127.79, 88.76, 82.38, 76.17, 74.26, 68.30, 62.87. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{12}H_{16}O_5SNa^+$: 295.1, found: 295.3.

Phenyl-3-benzoyl-1-thio-*β*-*L*-mannopyranoside (V)

Compound **IV** (0.50 g, 1.84 mmol) was dissolved in 12 ml of THF/water (19:1), then $(CH_3)_2SnCl_2$ (0.02 g, 0.092 mmol) and DIPEA (0.74 ml, 3.68 mmol) were sequentially added. After 5 mins of vigorous stirring, BzCl (0.28 g, 2.02 mmol) was added and the solution stirred for 6 h. Upon disappearance of starting material, the reaction was stopped with 3% HCl solution (20 ml) and extracted thrice with ethyl acetate (40ml each). The organic layers were combined and dried over anhydrous sodium sulphate and concentrated under vacuum. The residue was then purified by flash chromatography using ethyl acetate hexane (6.5:3.5) to give **V** (0.27 g, 0.74 mmol) as a white foam. Yield was 40 %. ^{1}H NMR (300 MHz, MeOD) δ 8.27 – 7.15 (m, 10H, aromatic protons), 5.17 (d, J = 1.1 Hz, 1H, H-1), 5.08 (dd, J = 9.8, 3.3 Hz, 1H, H-3), 4.42 (dd, J = 3.4, 1.0 Hz, 1H, H-2), 4.11 (dd, J_1 = 9.8 Hz, J_2 = 9.8 Hz 1H, H-4), 3.96 (dd, J = 12.0, 2.4 Hz, 1H, H-6a), 3.83 (dd, J = 12.0, 5.5 Hz, 1H, H-6b), 3.51 (ddd, J = 9.8, 5.5, 2.4 Hz, 1H, H-5). ^{13}C NMR (75 MHz, MeOD) δ 167.82, 136.86, 134.35, 131.51, 131.28, 130.91, 130.08, 129.52, 128.00, 88.66, 82.52, 79.06, 71.85, 65.71, 62.83. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{19}H_{20}O_6SNa^+$: 399.1, found: 399.2

Phenyl-3-benzoyl-4,6-benzylidene-1-thio-*β*-*L*-mannopyranoside (VI)

Compound **V** (0.27 g, 0.73 mmol) was dissolved in 10 ml acetonitrile. To this solution was added camphorsulfonic acid (0.042 g, 0.18 mmol) and benzaldehyde dimethyl acetal (0.13 g, 0.88 mmol) sequentially under argon atmosphere. The mixture was stirred for 30 mins and stopped with trimethylamine,. concentrated under vacuum and purified by flash chromatography using ethyl acetate/ hexane (3:7) to give **VI** (0.29 g, 0.62 mmol) as a white solid in 85 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 - 7.23 (m, 15H, aromatic protons), 5.63 (s, 1H, benzylidene CH), 5.34 (dd, *J* = 10.2, 3.3 Hz, 1H, H-3), 5.10 (s, 1H, H-1), 4.61 (d, *J* = 3.3 Hz, 1H, H-2), 4.49 – 4.30 (m, 2H, H-4, H-6a), 3.98 (m, 1H, H-6b), 3.68 – 3.59 (m, 1H, H-5), 2.59 (br s, 1H, C_2-OH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.83, 137.06, 133.47, 131.93, 129.92, 129.51, 129.22, 129.08, 128.51, 128.25, 128.05, 126.14, 101.83, 88.24, 75.41, 73.69, 71.79, 71.12, 68.49. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{26}H_{24}O_6SNa^+$: 487.1, found: 486.7

Phenyl-3-benzoyl-4,6-benzylidene-2-methylsulphonyl-2-deoxy-1-thio-*β*-*L*-mannopyranoside (6)

To synthesize **6,** compound **VI** (0.093 g, 0.20 mmol) and methanesulphonyl chloride (0.069 g, 0.60 mmol) were dissolved in 10 ml of pyridine and stirred for 18 h. The reaction was stopped by addition of 5 ml methanol, concentrated under vacuum, and the residue purified by flash chromatography using ethyl acetate/ hexane (1:4) to give **6** (0.081 g, 0.15 mmol) as a white solid. Yield was 75 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 – 7.23 (m, 15H, aromatic protons), 5.65 (s, 1H, benzylidene *CH*), 5.56 (dd, *J* = 3.2, 1.1 Hz, 1H, H-2), 5.47 (dd, *J* = 10.4, 3.1 Hz, 1H, H-3), 5.15 (d, *J* = 1.2 Hz, 1H, H-1), 4.46 – 4.28 (m, 2H, H-4, H-6a), 3.93 – 4.01 (m, 1H, H-6b), 3.66 - 3.71 (m, 1H), 3.26 (s, 3H, mesylate *CH_3*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.00, 136.81, 133.39, 132.42, 132.28, 130.23, 129.48, 129.29, 129.18, 128.76, 128.38, 128.28, 126.16, 101.91, 86.83,

80.18, 75.01, 72.14, 71.12, 68.27, 39.39. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{27}H_{26}O_8S_2Na^+$: 565.1, found: 565.5.

Phenyl-2-azido-2-deoxy- 3-benzoyl-4,6-benzylidene -1-thio-*β-L*-glucopyranoside (7)

Compound **6** (0.67 g, 1.24 mmol) was treated with sodium azide (0.81 g, 12.4 mmol) in 10 ml of anhydrous *N,N*-dimethyl formamide under argon atmosphere at 70 °C for 24 h. The reaction mixture was then concentrated under vacuum, diluted with ethyl acetate, and solid sodium azide residue filtered. The organic layer was re-concentrated, and the residue purified by flash chromatography using ethyl acetate/dichloromethame/hexane mixture (2:1:8) to give **7** (0.21 g, 0.43 mmol) as a white precipitate. Yield was 36 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 – 7.22 (m, 15H), 5.68 (dd, J_1, J_2 = 9.5 Hz, 1H, H-3), 5.57 (s, 1H, benzylidene *CH*), 4.81 (d, *J* = 10.1 Hz, 1H, H-1), 4.47 (dd, *J* = 10.4, 4.8 Hz, 1H, H-6a), 3.98 – 3.57 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.48, 133.71, 130.01, 129.43, 128.91, 128.63, 128.33, 127.65, 127.37, 126.29, 101.45, 87.20, 78.55, 73.75, 70.81, 68.47, 64.15. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{26}H_{23}N_3O_5SNa^+$: 512.1, found: 512.4

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(2'azido-2'-deoxy-3'-benzoyl-4',6'-benzylidene-α-L-glucopyranosyl)-sn-glycerol (9)

The fully protected glycoside donor **7** (0.10 g, 0.20 mmol) and the glycoside acceptor **8** (0.086 g, 0.26 mmol) were dissolved in 15 ml of dichloromethane under argon atmosphere, followed by the simultaneous addidtion of silver triflate (0.01 g, 0.04 mmol) and *N*-iodosuccinimide (0.067 g, 0.30 mmol). The reaction was stirred at room temperature for 2 h after which it was stopped with 5 ml saturated sodium thiosulphate solution and washed with saturated sodium bicarbonate (×3),

water (×1), and brine (×1). The organic layer was dried over anhydrous sodium sulphate and concentrated under vacuum. The residue was purified by flash chromatography using ethyl acetate/dichloromethame/hexane mixture (1:1:8) to give α-anomer, **9** (0.068 g, 0.096 mmol) as a white solid in 48 % yield, containing < 10 % of the corresponding β-anomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 – 7.14 (m, 10H, aromatic protons), 5.89 (dd, $J_1 = J_2 = 9.9$, Hz, 1H, H-3), 5.56 (s, 1H, benzylidene *CH*), 5.11 (d, $J = 3.6$ Hz, 1H, H-1), 4.37 (dd, $J = 10.3, 4.8$ Hz, 1H), 4.13 - 4.07 (m, 1H), 3.72-3.91 (m, 3H), 3.66 – 3.26 (m, 10H, H-2), 1.67 – 1.48 (m, 2H), 1.29 (br s, 26H, lipid tail), 0.88 (t, $J = 7.6$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.47, 136.90, 133.25, 129.94, 129.09, 128.40, 128.19, 127.56, 126.17, 101.67, 99.28, 79.67, 79.06, 71.88, 69.62, 69.50, 68.86, 68.11, 62.92, 61.90, 58.27, 31.96, 29.73, 29.52, 29.40, 26.14, 22.72, 14.15. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{40}H_{59}N_3O_8Na^+$: 732.4, found: 732.6

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(2'azido-2'-deoxy-4',6'-benzylidene-α-L-glucopyranosyl-*sn*-glycerol (VII)

Compound **9** (0.068 g, 0.096 mmol) was treated with sodium methoxide in 10 ml methanol and stirred for 3 h. The reaction was stopped with acidic ion exchange resin, filtered, concentrated, and purified by flash chromatography using ethyl acetate/dichloromethane/hexane mixture (3:1:7) to give **VII** (0.043 g, 0.07 mmol) as a white in 72 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.55 – 7.35 (m, 5H, aromatic protons), 5.56 (s, 1H, benzylidene *CH*), 4.96 (d, $J = 3.6$ Hz, 1H, H-1), 4.29 – 4.19 (m, 2H), 3.92 (dd, $J = 9.8, 4.8$ Hz, 1H), 3.84 – 3.39 (m, 12H), 3.28 (dd, $J = 10.0, 3.6$ Hz, 1H, H_2), 1.62 – 1.53 (m, 2H), 1.32 (br s, 26H, lipid tail), 0.90 (t, $J = 6.6$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.67, 136.99, 129.36, 128.38, 126.30, 123.77, 102.11, 98.83, 81.93,

79.08, 71.87, 69.69, 68.86, 68.61, 67.91, 63.11, 62.50, 58.21, 31.95, 29.72, 29.64, 29.51, 29.38, 26.13, 22.71, 14.14. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{33}H_{55}N_3O_8Na^+$: 644.4, found: 644.5

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(2'azido-2'-deoxy-α-L-glucopyranosyl)-sn-glycerol (VIII)

Compound **VII** (0.043 g, 0.07 mmol) was dissolved in acetic acid water mixture (4:1) and stirred at 60 °C for 5 h. The mixture was concentrated and purified with ethyl acetate/ hexane mixture (9:1) to give **VIII** (0.027 g, 0.053 mmol) as white solid. Yield was 75 %. ^{1}H NMR (300 MHz, MeOD) δ 4.92 (d, *J* = 3.5 Hz, 1H, H-1), 3.94 – 3.66 (m, 2H), 3.66 – 3.55 (m, 5H), 3.55 – 3.36 (m, 8H), 3.10 (dd, *J* = 10.5, 3.5 Hz, 1H, H-2), 1.71 – 1.51 (m, 2H), 1.32 (s, 26H, lipid tail), 0.88 (t, *J* = 6.6 Hz, 3H, terminal lipid *CH_3*). ^{13}C NMR (75 MHz, MeOD) δ 99.77, 80.55, 74.03, 72.69, 72.42, 72.07, 71.20, 68.17, 64.53, 62.45, 58.37, 33.12, 30.84, 30.80, 30.52, 27.29, 23.78, 14.51. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{26}H_{51}N_3O_7Na^+$: 540.4, found: 540.1

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(2'-amino-2'deoxy-α-L-glucopyranosyl)-sn-glycerol (2)

To a solution of compound **VIII** (0.027 g, 0.053 mmol) in a 12 ml THF/water mixture (5:1) was added 2ml of 1M trimethylphosphine in THF. The reaction was stirred at room temperature for 2 h after which it was concentrated and was purified by C-18 column using gradient elution of water/methanol, to give **2** (0.016 g, 0.033 mmol) as a white solid in 63 % yield. Compound **2** has slight traces of the β- anomer. ^{1}H NMR (300 MHz, MeOD) δ 4.71 (d, *J* = 3.6 Hz, 1H, H-1), 3.76 – 3.66 (m, 1H), 3.62 (dd, *J* = 11.8, 5.3 Hz, 1H), 3.55 – 3.27 (m, 12H), 3.26 – 3.16 (m, 2H), 2.50 (dd, *J* = 9.8, 3.5 Hz, 1H, H-2), 1.43 – 1.53 (m, 2H), 1.22 (s, 26H, lipid tail), 0.83 (t, *J* = 6.5 Hz, 3H, terminal lipid *CH_3*). ^{13}C NMR (75 MHz, MeOD) δ 100.59, 80.50, 76.34, 74.22, 72.70, 71.88, 71.41, 67.92, 62.68, 58.26, 57.28, 33.11, 30.80, 30.65, 30.51, 27.29, 23.77, 14.49.

MALDI-HRMS: *m/e* $[M+Na]^+$ calcd for $C_{26}H_{53}NO_7Na^+$: 514.3720, found: 514.3729. Elemental Analysis: calcd: C, 63.51; H, 10.86; N, 2.85, found: C, 63.05; H, 11.01; N, 2.86

Phenyl-3-benzoyl-4-benzyl-1-thio-β-*L*-mannopyranoside (IX)

To a solution of **VI** (0.46 g, 1.00 mmol) in anhydrous dichloromethane (10 ml) were added 150 μL (1.57 mmol) of 1M borane tetrahydrofuran complex solution and trimethylsilyl trifluoromethanesulfonate (0.36 g, 1.62 mmol). The reaction was stirred at room temperature for 3 h, stopped with 1 ml trimethylamine, and concentrated under vacuum. The residue was purified by flash chromatography using hexane/dichloromethane/ethyl acetate mixture (5:1:4) to give **IX** (0.38 g, 0.80 mmol) as a white solid in 80 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 – 7.96 (m, 2H, aromatic protons), 7.71 – 7.09 (m, 13H, aromatic protons), 5.27 (dd, *J* = 9.8, 3.2 Hz, 1H, H-3), 5.04 (d, *J* = 1.1 Hz, 1H, H-1), 4.84 – 4.64 (m, 2H, benzyl CH_2), 4.50 (dd, *J* = 1.1, 3.2 Hz, 1H, H-2), 4.26 (dd, *J* = 9.8, 9.7 Hz, 1H, H-4), 3.98 (d, *J* = 12.2 Hz, 1H), 3.78 - 3.89 (m, 1H), 3.54 (ddd, *J* = 9.7, 4.2, 2.6 Hz, 1H, H-5), 2.73 (br s, 1H, C_2-OH), 2.42 (br s, 1H, C_6-OH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.74, 137.54, 133.61, 133.50, 131.57, 129.83, 129.53, 129.22, 128.60, 128.44, 128.07, 127.88, 87.20, 80.04, 77.29, 75.23, 72.17, 71.03, 61.96. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{26}H_{26}O_6SNa^+$: 489.1, found: 488.8

Phenyl-3-benzoyl-4-benzyl-2,6-dimethysulphonyl-2,6-dideoxy-1-thio-β-*L*-mannopyranoside (10)

Compound **IX** (0.38 g, 0.80 mmol), methanesulphonyl chloride (0.28 mg, 2.45 mmol), and catalytic DMAP (0.05 g) were dissolved in 10 ml pyridine. The reaction was stirred under argon atmosphere for 24 h and stopped with 5ml methanol. The mixture was concentrated under

vacuum, re-dissolved in dichlromethane and washed with 3% HCl solution (×2), saturated sodium bicarbonate solution (×2), and water (×1). The organic layer was dried over anhydrous sodium sulphate, concentrated, and purified by flash chromatography using hexane/dichloromethane/ethyl acetate mixture (5:2:3) to give **10** (0.40 g, 0.64 mmol) as an off white solid. Yield was 80 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 – 8.08 (m, 2H aromatic protons), 7.74 – 7.05 (m, 13H, aromatic protons), 5.39 – 5.47 (m, 2H, H_2, H_3), 5.11 (dd, *J* = 1.1 Hz 1H, H-1), 4.79 – 4.60 (m, 2H, benzyl CH_2), 4.49 (dd, *J* = 11.8, 1.9 Hz, 1H, H-6a), 4.39 (dd, *J* = 11.7, 4.6 Hz, 1H, H-6b), 4.13 (dd, *J* = 10.5, 8.5 Hz, 1H), 3.75 – 3.66 (m, 1H, H-5), 3.18 (s, 3H, mesylate CH_3), 3.07 (s, 3H, mesylate CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.70, 149.88, 136.94, 135.99, 133.69, 132.34, 131.98, 130.12, 129.47, 129.11, 128.65, 128.57, 128.23, 123.79, 85.27, 79.72, 77.47, 75.50, 74.36, 71.78, 68.44, 39.31, 37.96. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{28}H_{30}O_{10}S_3Na^+$: 645.1, found: 645.3.

Phenyl-3-benzoyl-4-benzyl-2,6-diazido-2,6-dideoxy-1-thio-*β*-*L*-glucopyranoside (11)

To a solution of compound **10** (0.40 g, 0.64 mmol) in 10 ml *N,N*-dimethyformamide was added sodium azide (0.86 g, 13.2 mmol). The reaction was stirred under argon atmosphere at 90 °C for 24 h. The mixture was then concentrated, re-dispersed in ethylacetate and the sodium azide residue filtered. The organic solvent was re-concentrated and purified by flash chromatography using ethyl acetate/dichloromethame/hexane mixture (1:1:8) to give the fully protected glycoside donor **11** (0.13 g, 0.26 mmol) as a white solid. Yield was 41 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 – 8.06 (m, 2H, aromatic protons), 7.76 – 7.67 (m, 2H, aromatic protons), 7.67 – 7.46 (m, 5H), 7.47 – 7.11 (m, 6H, aromatic protons), 5.50 (dd, *J* = 9.4 Hz, 1H, H_3), 4.64 (d, *J* = 10.1 Hz, 1H, H-1), 4.61 – 4.50 (m, 2H, Benzyl CH_2), 3.75 (dd, $J_1 = J_2$= 9.3 Hz, 1H, H_4), 3.70 – 3.59 (m,

2H, H-5, H-6a), 3.51 (dd, *J* = 10.1, 9.4 Hz, 1H, H-2), 3.44 (dd, *J* = 13.4, 4.5 Hz, 1H, H-6b). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.42, 136.93, 134.60, 133.62, 130.27, 129.91, 129.30, 129.25, 129.15, 129.01, 128.65, 128.49, 128.20, 128.16, 127.60, 127.24, 86.15, 78.35, 76.53, 75.97, 75.02, 63.47, 51.14. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{26}H_{24}N_6SNa^+$: 539.2, found: 539.1.

Synthesis of Compounds 12a/b

The fully protected glycoside donor **11** (0.14 g, 0.26 mmol) and glycoside acceptor **8** (0.13 g, 0.40 mmol) were dissolved in 15 ml dichloromethane, followed by the simultaneous addition of silvertriflate (0.013 g, 0.05 mmol) and *N*-iodosuccinimide (0.12 g, 0.52 mmol). The reaction was stirred under argon atmosphere for 2 h, stopped with saturated sodium thiosulphate solution, and washed successively with 25 ml each of saturated sodium bicarbonate (×3), water (×1) and brine (×1). The organic layer was then dried over anhydrous sodium sulphate, concentrated, and purified by flash chromatography using ethyl acetate/dichloromethame/hexane mixture (1:1:8) to give α- and β- anomers, **12a** (0.066 g, 0.09 mmol) and **12b** (0.022.g, 0.03 mmol) respectively. Reaction yield was 45 %. NMR data of compounds **12a/b** are below

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(3'-benzoyl-4'-benzyl-2',6'-diazido-2',6'-dideoxy-α-L-glucopyranosyl)-sn-glycerol (12a)

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (d, *J* = 7.2 Hz, 2H, benzoyl *o*- protons) 7.67 – 7.56 (m, 1H, benzoyl *p*- proton), 7.49 (t, *J* = 7.6 Hz, 2H, benzoyl *m*- protons), 7.34 – 7.10 (m, 5H, benzyl aromatic protons), 5.85 (dd, *J* = 10.7, 8.9 Hz, 1H, H-3), 5.09 (d, *J* = 3.5 Hz, 1H, α-H-1), 4.61 - 4.55 (m, 2H, benzyl CH_2), 4.15 – 4.02 (m, 1H, H-5), 3.91 – 3.77 (m, 2H), 3.71 (dd, *J* = 10.3, 3.9 Hz, 1H), 3.67 – 3.39 (m, 10H), 3.31 (dd, *J* = 10.7, 3.5 Hz, 1H, H-2), 1.55 - 1.66 (m, 2H), 1.28 (br

s, 26H, lipid tail), 0.91 (t, *J* = 7.5 Hz, 3H, terminal lipid *CH₃*).[13]C NMR (75 MHz, $CDCl_3$) δ 165.44, 137.10, 133.42, 129.88, 129.51, 128.54, 128.43, 128.07, 98.39, 79.08, 76.76, 74.97, 72.57, 71.87, 70.22, 69.69, 68.14, 61.46, 58.33, 51.04, 31.95, 29.73, 29.69, 29.64, 29.52, 29.39, 26.13, 22.72, 14.15. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{40}H_{60}N_6O_7Na^+$: 759.5, found: 759.4

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(3'-benzoyl-4'-benzyl-2',6'-diazido-2',6'-dideoxy-β-L-glucopyranosyl)-sn-glycerol (12b)

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (d, *J* = 7.2 Hz, 2H, benzoyl *o*- protons), 7.65 – 7.56 (m, 1H, benzoyl *p*- proton), 7.50 (t, *J* = 7.6 Hz, 2H, benzoyl *m*- protons), 7.32 – 7.06 (m, 5H, benzyl aromatic protons), 5.34 (dd, *J* = 10.4, 8.8 Hz, 1H, H-3), 4.59 (d, *J* = 7.6 Hz, 1H, β-H-1), 4.57 – 4.45 (m, 2H, benzyl *CH₂*), 4.08 (dd, *J* = 10.7, 3.2 Hz, 1H), 3.78 – 3.67 (m, 2H), 3.67 – 3.28 (m, 12H), 1.57 – 1.62 (m, 2H), 1.31 (br s, 26H, lipid tail), 0.90 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.35, 136.82, 133.51, 129.89, 129.40, 129.06, 128.58, 128.47, 128.20, 128.16, 102.27, 79.36, 76.47, 74.93, 74.65, 74.52, 71.83, 69.77, 69.69, 64.48, 58.00, 51.03, 31.95, 29.72, 29.68, 29.63, 29.57, 29.52, 29.49, 29.38, 26.11, 26.07, 22.71, 14.14. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{40}H_{60}N_6O_7Na^+$: 759.5, found: 759.3

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(4'-benzyl-2',6'-diazido-2',6'-dideoxy-α-L-glucopyranosyl)-sn-glycerol (X)

Compound **12a** (0.066 g, 0.09 mmol) was treated with sodium methoxide in methanol (10 ml) and stirred for 3 h. The reaction was stopped with acidic ion exchange resin, filtered, concentrated, and purified by flash chromatography using ethyl acetate/dichloromethane/hexane mixture (2:1:7) to give **X** (0.04 g, 0.063 mmol) as a white solid. Yield was 70 %. ^{1}H NMR (300

MHz, $CDCl_3$) δ 7.48 – 7.25 (m, 5H, aromatic proton), 4.95 (d, *J* = 3.6 Hz, 1H, α-H-1), 4.80 (dd, *J* = 11.4 Hz, 2H, benzyl *CH_2*), 4.12 (dd, *J* = 10.3, 8.5,Hz, 1H, H-3), 3.98 – 3.85 (m, 1H, -***CH***-*O*-*CH_3*), 3.78 (dd, *J* = 9.1, 3.8 Hz, 1H), 3.69 – 3.35 (m, 12H), 3.21 (dd, *J* = 10.3, 3.6 Hz, 1H, H-2), 2.47 (br s, 1H, C_3 - OH), 1.50 – 1.62 (m, 2H), 1.35 (s, 26H, lipid tail), 0.91(t, *J* = 7.6 Hz, 3H, terminal lipid *CH_3*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.80, 128.74, 128.26, 128.01, 97.88, 79.07, 78.92, 75.14, 71.86, 71.79, 70.21, 69.78, 67.81, 63.06, 58.21, 51.20, 31.95, 29.72, 29.68, 29.63, 29.51, 29.38, 26.12, 22.71, 14.14. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{33}H_{56}N_6O_6Na^+$: 655.4, found: 655.6

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(4'-benzyl-2',6'-diazido-2',6'-dideoxy-β-L-glucopyranosyl)-sn-glycerol (XI)

Compound **12b** (0.022 g, 0.03 mmol) was treated similarly as **12a** to give **XI** (0.013 g, 0.021 mmol) as a white solid. Yield was 70%. NMR data of **XI**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 – 7.24 (m, 5H, aromatic protons), 4.78 (m, 2H, benzyl *CH_2*) 4.42 (d, *J* = 7.9 Hz, 1H, β- H-1), 4.06 (dd, *J* = 10.5, 3.0 Hz, 1H), 3.68 (dd, *J* = 10.6, 5.4 Hz, 1H), 3.62 – 3.31 (m, 14H), 2.51 (br s, 1H, *C_3- OH*), 1.52 – 1.61 (m, 2H), 1.34 (m, 26H), 0.89 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.67, 128.67, 128.23, 128.16, 102.14, 79.33, 77.87, 75.26, 74.96, 74.74, 71.81, 69.85, 69.67, 66.40, 58.02, 51.27, 31.95, 29.72, 29.64, 29.51, 29.38, 26.11, 22.71. ES-MS: *m/z* $[M+Na]^+$ calcd for $C_{33}H_{56}N_6O_6Na^+$: 655.4, found: 655.8.

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(4'-benzyl-2',6'-diamino-2',6'-dideoxy-α-L-glucopyranosyl)-sn-glycerol (4)

Compound **X** (0.04 g, 0.063 mmol) was treated similarly as **2** to give **4** (0.026 g, 0.044 mmol) as a white solid. Yield was 60%. ^{1}H NMR (300 MHz, MeOD) δ 7.41 – 7.07 (m, 5H, aromatic proton), 4.85 (d, *J* = 11.3, 1H, benzyl *CH*$_2$), 4.68 (d, *J* = 3.6, 1H, α-H-1), 4.55 (dd, *J* = 11.3, 1H, benzyl *CH*$_2$), 3.72 – 3.60 (m, 1H, -***CH***-*O*-*CH*$_3$), 3.58 – 3.28 (m, 12H), 3.07 (dd, *J* = 10.0, 1.5 Hz, 1H, H-6b), 2.59 – 2.46 (m, 2H, , H-2, H-6a), 1.55 – 1.36 (m, 2H, -*OCH*$_2$***CH***$_2$), 1.19 (m, 26H), 0.80 (t, *J* = 6.7, 3H, terminal lipid *CH*$_3$). ^{13}C NMR (75 MHz, MeOD) δ 139.99, 129.31, 128.82, 100.36, 80.83, 80.40, 76.80, 75.66, 73.08, 72.71, 71.14, 67.78, 58.27, 57.68, 43.72, 33.12, 30.83, 30.63, 30.52, 27.29, 23.78, 14.51. MALDI-HRMS: *m/z* $[M+Na]^+$ calcd for $C_{33}H_{60}N_2O_6Na^+$: 603.4349, found: 603.4260

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(2',6'-diamino-2',6'-dideoxy-β-L-glucopyranosyl)-sn-glycerol (3)

To a solution of compound **4** (0.017 g, 0.03 mmol) in methanol was added palladium hydroxide on carbon (0.02 g), and the reaction was stirred under hydrogen atmosphere for 15 h. The catalyst was filtered, concentrated, and purified by C-18 column using gradient elution of water/methanol to give **3** (0.008 g, 0.017 mmol) as a white solid. Yield is 58 %. ^{1}H NMR (500 MHz, MeOD) δ 4.77 (d, *J* = 3.7 Hz, 1H, α-H-1), 3.83 – 3.73 (m, 1H, -***CH***-*O*-*CH*$_3$), 3.61 – 3.39 (m, 11H), 3.14 (dd, *J* = 9.8, 8.8 Hz, 1H, H-3), 2.97 (dd, *J* = 13.4, 3.1 Hz, 1H, H-6a), 2.71 (dd, *J* = 13.4, 7.1 Hz, 1H, H-6b), 2.56 (dd, *J* = 9.9, 3.7 Hz, 1H, H-2), 1.63 – 1.49 (m, 2H, -*OCH*$_2$***CH***$_2$-), 1.29 (m, 26H, lipid tail), 0.89 (t, *J* = 6.9 Hz, 3H, terminal lipid *CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 99.09, 79.03, 74.76, 72.54, 72.06, 71.22, 69.85, 66.54, 56.82, 55.91, 42.38, 31.64, 29.34, 29.32, 29.03, 25.83, 22.30, 12.99. MALDI-HRMS: *m/e* $[M+Na]^+$ calcd for

$C_{26}H_{54}N_2O_6Na^+$: 513.3880, found: 513.3883. Elemental Analysis: calcd: C, 63.64; H, 11.09; N, 5.71, found: C, 63.67; H, 11.11; N, 5.68

1-*O*-Hexadecyl-2-*O*-methyl-3-O-(4'-benzyl-2',6'-diamino-2',6'-dideoxy-β-L-glucopyranosyl)-sn-glycerol (5)

Compound **XI** (0.013 g, 0.021 mmol) was treated similarly as **2** to give **5** (0.007 g, 0.013 mmol) as a white solid. Yield was 60 %. ^{1}H NMR (500 MHz, MeOD) δ 7.43 – 7.20 (m, 5H, aromatic proton), 4.78 (dd, *J* = 11.2 Hz, 2H, benzyl *CH*$_2$), 4.23 (d, *J* = 7.9 Hz, 1H, β- H-1), 3.93 – 3.98 (m, 1H, -***CH***-*O*-*CH*$_3$), 3.67 – 3.45 (m, 10H, -*OCH*$_3$, H-5), 3.36 – 3.11 (m, 2H), 2.95 (dd, *J* = 13.4, 2.8 Hz, 1H, H-6b), 2.72 – 2.55 (m, 2H, H-2, H-6a), 1.58 – 1.54 (m, 2H, -*OCH*$_2$***CH***$_2$-), 1.28 (m, 26H, lipid tail), 0.89 (t, *J* = 6.8 Hz, 3H, terminal lipid *CH*$_3$). ^{13}C NMR (125 MHz, MeOD) δ 138.43, 127.95, 127.38, 103.65, 79.45, 79.33, 76.61, 75.73, 74.24, 71.24, 69.66, 68.81, 57.37, 56.69, 42.42, 31.65, 29.36, 29.33, 29.15, 29.05, 25.80, 22.31, 13.01. MALDI-HRMS: *m/z* $[M+Na]^+$ calcd for $C_{33}H_{60}N_2O_6Na^+$: 603.4349, found: 603.4351

10.2 Biological methods

10.2.1 Effect of GAELs on the viability of epithelial cancer cell lines

The cell lines were cultured from frozen stocks originally obtained from ATCC. MDA-MB-231, JIMT-1, and DU-145 were grown in DMEM medium supplemented with 10% FBS, BT-474 cells in DMEM/F12 medium supplemented with 10% FBS, MiaPaCa-2 in DMEM supplemented with 10% FBS and 2.5% horse serum, and PC-3 cells cultured in F12K medium supplemented

with 10% FBS. All the media contained penicillin/streptomycin. The effects of the GAELs on the viability of the various epithelial cancer cell lines was determined as previously described.[4–7] Briefly, equal numbers of the cells were dispersed into 96-well plates. After 24 h, the cells were incubated with the compounds (0-30 μM) for 48 h. At the end of the incubation, MTS reagent (20% v/v; Promega) was added and the plates were incubated for 1-4 h in a CO_2 incubator. The OD_{490} was read with a plate reader (Molecular Devices). Wells with media but no cells were treated in similar fashion and the values utilized as blank. The results represent the mean ± standard deviation of 6 independent determinations.

10.2.2 Demonstration of caspase- independent mode of cell death

JIMT-1 and DU-145 cells were grown in DMEM medium containing penicillin/streptomycin and supplemented with 10% FBS. Equal numbers of the cells were dispersed into 96-well plates, and after 4 h, the cells were treated with pan-caspase inhibitor QVD-OPh (40 μM). After 20 h, the cells were incubated with varying concentrations of the compounds (0 – 9 μM) for additional 48 h, and at the end, MTS reagent (20% v/v) was added and the plates were further incubated for 1-4 h in a CO_2 incubator. The OD_{490} was read with a plate reader (Molecular Devices). Wells with media but no cells were treated in similar fashion and the values utilized as blank. The results represent the mean ± standard deviation of 6 independent determinations.

10.2.3 Determination of membranolytic effects of GAELs

JIMT-1 and DU-145 were grown in DMEM medium supplemented with 10% FBS and antibiotics, penicillin/streptomycin. Equal numbers of the cells were dispersed into 96-well plates. After 24 h, the cells were incubated with varying concentrations of compounds **4** and **5** (4

- 6 μM) for 5 - 6 h. Subsequently, the cell membrane impermeant DNA staining dye, ethidium homodimer-1 (EthD-1, Molecular Probes) at a final concentration of 2 μM was added and the cells analysed by fluoresecence microscopy.[13] EthD-1 staining was compared to negative controls with no treatment and positive control treated with 0.01% Triton X-100 for 10 min.

10.2.4 Hemolytic assay

The hemolytic activity of the GAEL analogs was evaluated using ovine erythrocyte. Sheep whole blood was collected from a slaughter house into a container containing disodium EDTA in a buffered saline (10mM Tris, 150 mM NaCl, pH 7.4). The erythrocytes were prepared and wash with buffered saline as previously reported.[8,9] For the assay, the erythrocyte suspension, varying amounts of the GAEL drugs, the saline buffer and the appropriate amount of the vehicle used for dissolving the compounds were pipetted to an Eppendorf tubes to give a final volume of 1500 μL and cell density of 2.5×10^8 cells/ml. The suspensions were incubated with gentle shaking in Eppendorf thermomixer for 30 mins. The eppendorf tubes were cooled in ice-water and centrifuged at 2000 g and 4 $^{\circ}$C for 5 min. 200 μL of the supernatant was dissolved in 1800 μL of 0.5% NH_4OH and the optical density (OD) was recorded using 1 mL cuvette at 540 nm in a spectrophotometer. For 0% hemolysis (negative control), buffer and vehicle used to dissolve the drug were added instead of the drug, while and for 100% hemolysis (positive control), 1% NH_4OH was used. % hemolysis was calculated using the optical density (OD) values as shown below:

$$\% \text{ hemolysis} = (X - 0\%)/ (100\% - 0\%)$$

X is OD values of the drugs at varying concentration.

10.2.5 Isolation of cancer stem cells from BT-474 cell lines and determination of the effect of GAELs on the viability of the cancer stem cells.

A population enriched in BT-474 breast cancer stem cells was obtained by staining the cells for aldehyde dehydrogenase using the Aldefluor assay kit from Stem Cell Technologies (Vancouver, BC, Canada) according to the instruction of the manufacturer, with the appropriate controls. The stained cells were sorted from the bulk population by flow cytometry on a 4 laserMoFloXPP high speed/pressure cell sorter. The cells were pelleted by centrifugation and resuspended into ultra-low adhesion plates in mammocult medium (Stem Cell Technologies). The dishes were incubated at 37 °C in a CO_2 incubator for 5 days for spheroid formation.

The spheres are separated from single cells with a 40 µm nylon cell strainer. The spheres retained in the strainer were washed with PBS and trypsinised to obtain single cells. The cell numbers were counted with a Coulter ZM counter and the cells were dispersed into 96-well ultra-low adhesion plates in a volume of 100 µl, and incubated for 3 days to allow for formation of spheroids. Subsequently, the stock GAELs in ethanol were diluted to twice the final concentration in the media and a volume of 100 µl was added to each well. Wells with growth medium but no cells were treated similarly as the wells with cells. After a 3-day incubation, MTS reagent (20% v/v) was added to each well and the plates were incubated further for 1 - 4 h for formation of color. The OD_{490} were read in a Molecular Device absorbance plate reader and data extrapolated using the SpectroMax software.

10.2.6 Evaluation of the stability of L-glucosamine derived GAELs to glycosidases

A fraction containing α- and β-glycosidases (glucosidases, mannosidases, galactosidases and glucosaminidases) was prepared from beef liver as previously reported.[8,9] Fresh whole beef

liver was obtained from a slaughter house (Robert Farm, Winnipeg), the connective tissue covering the liver removed, and 100 g of the liver cut into small pieces and homogenized in 1 L of cold water (4 °C) in a blender for 1 min. The pH was adjusted to 4.8 using 1 M citric acid. The mixture was centrifuged and 0.20 M to 0.60 M saturated ammonium sulfate fraction precipitate was obtained from the supernatant. The fraction was dissolved in distilled water and dialyzed against water at 4 °C for 24 h, and against 0.05 M sodium citrate buffer, pH 5.0, for a further 24 h. Any precipitate that formed was discarded and the supernatant was stored at -20 °C. At this temperature the glycosidases are stable for many months.[9] The protein content of the fraction was 7.5 mg/ml, based on protein assay using precision red advanced protein assay reagent (Cytoskeleton Inc) as stipulated by the manufacturer.

The glycosidases activity of the fraction was assessed as previously described.[10] Briefly, the assay mixture (2 ml) consisted of 0.5 ml of 0.2 M citric acid-NaOH buffer, pH 4.4, 0.4 ml of 25 mM of *p*-nitrophenyl α-glucoside, 25 µL of enzyme solution, 0.225 ml of buffer solution used as vehicle for the enzyme, and 0.85 ml of distilled water. The final concentration of the substrate in the incubated mixture was 10 mM. After incubation at 38 °C for 1 h, 2 ml of 0.4 M glycine-NaOH buffer, pH 10.5, was added. The solution was centrifuged and the liberated *p*-nitrophenol was measured with spectrophotometer at 410 nm. The activity of the enzyme was calculated to be 19.26 µg/mg/hr.[10]

For the assay with pure enzyme, α-glucosidase enzyme purified from *Saccharomyces cerevisiae* was purchased from Sigma Aldrich, and the reaction carried out according to their protocol. Briefly, the assay mixture (2 ml) consisted of 1.7 ml of 67 mM KH_2PO_4, pH 6.8, 0.25 ml of 25 mM *p*-nitrophenyl α-glucoside, 0.05 ml of distilled water used as vehicle for the enzyme, and 0.25 unit of enzyme. After incubation at 37 °C for 1 h, 2 ml of 100 mM Na_2CO_3 was added. The

solution was centrifuged and the liberated *p*-nitrophenol measured with spectrophotometer at 410 nm.

To assess the stability of L-glucosamine GAELs **2** and **4** to the liver glycosidases fraction, the reaction mixture (50 µL) was composed of 7.5 µL of 0.2 M sodium citrate buffer, pH 4.4, 12.5 µL of 30 mM of the substrates, 25 µL of the glycosidase fraction in 0.05 M sodium citrate buffer, pH 5.0 and 5 µL of 10 % triton X-100 for solubilization of the substrate in the mixture. For the negative control (without enzyme), 25 µL of 0.05 M sodium citrate buffer, pH 5.0 were used. Triton X100 was established to have no effect on enzyme activity. A positive control using β-D-glucose-based GAEL was also investigated under identical conditions. After 1 h incubation, 50 µL of methanol was added to inactivate the enzyme, centrifuged, and the supernatant was analyzed using chromatographic techniques.

Since the L-GAELs of interest are alpha-anomers, we decided to investigate their stability to pure α-glucosidase enzyme alongside the liver extract cocktail. The reaction mixture (50 µL) was composed of 10 µL of 285 mM KH_2PO_4, pH 6.8, 10 µL of 30 mM of substrate, 30 µL of distilled water for solubilizing both substrates and enzyme, and 0.25 unit of enzyme. A negative control without enzyme and positive control using glucose-based D-GAEL was carried out similarly as the test assay. After 1 h incubation, 50 µL of methanol was added to inactivate the enzyme, centrifuged, and the supernatant analyzed using chromatographic techniques. All experiments were carried out in quadruples. Thin-layer chromatography was used for qualitative determination of the presence of enzymatic degradation products of all compounds, while additional HPLC analysis was done for compound **4** that has a chromophore.

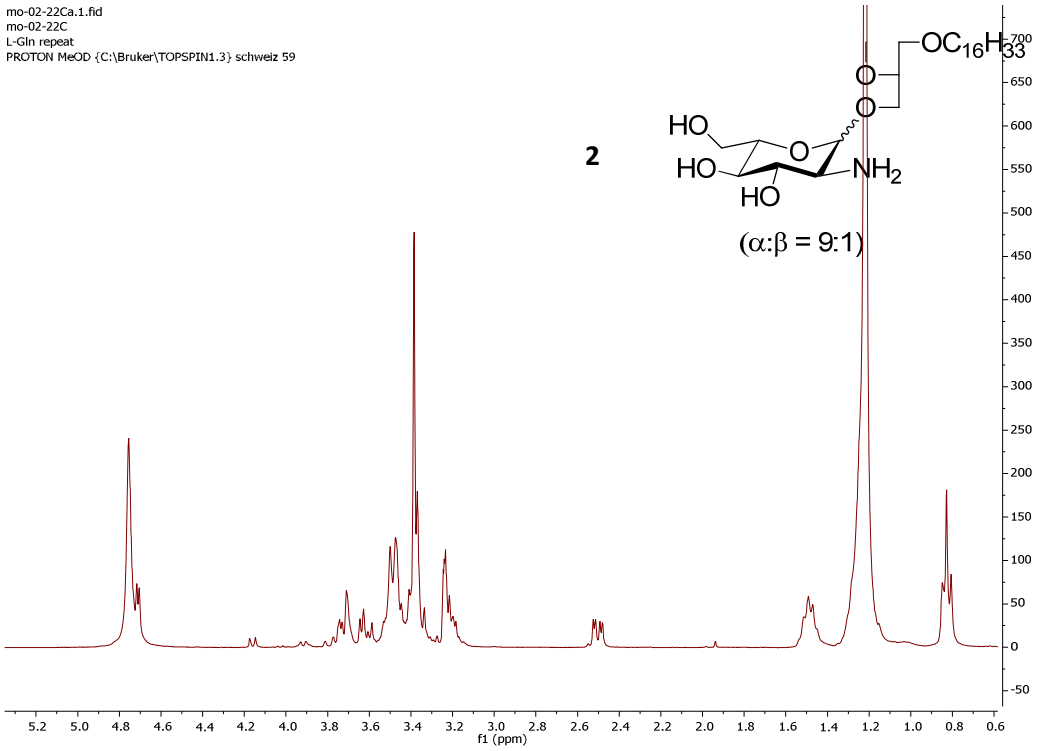
The results of the study showed that the β-D-glucose-based GAEL was completely cleaved by the liver extract but not the pure α-glucosidase enzyme, while compounds **2** and **4** were resistant

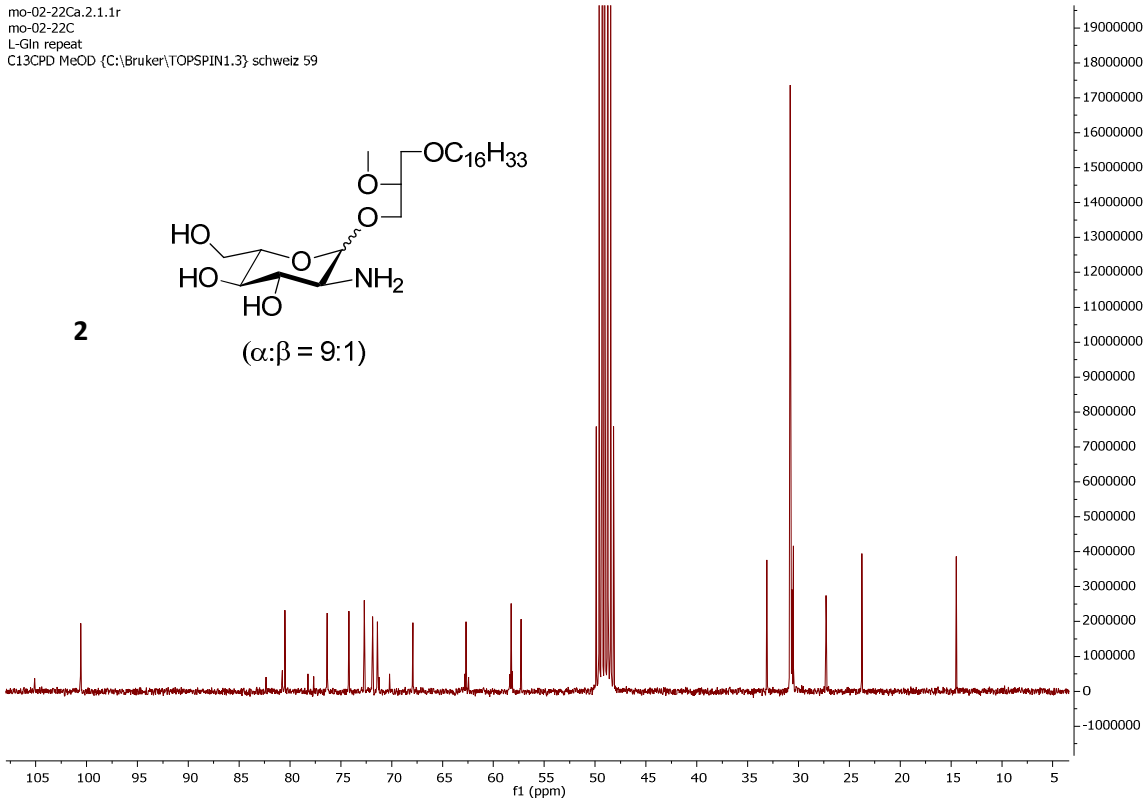
to both animal and pure glycosidases-catalyzed hydrolysis. The HPLC analysis of 4 before and after the reaction are indeed identical, showing that the compound is still intact and has no degradation product. This outcome confirms our hypothesis of unnatural L-sugar-based GAELs being resistant to metabolic degradation, especially glycosidases-catalyzed hydrolysis.

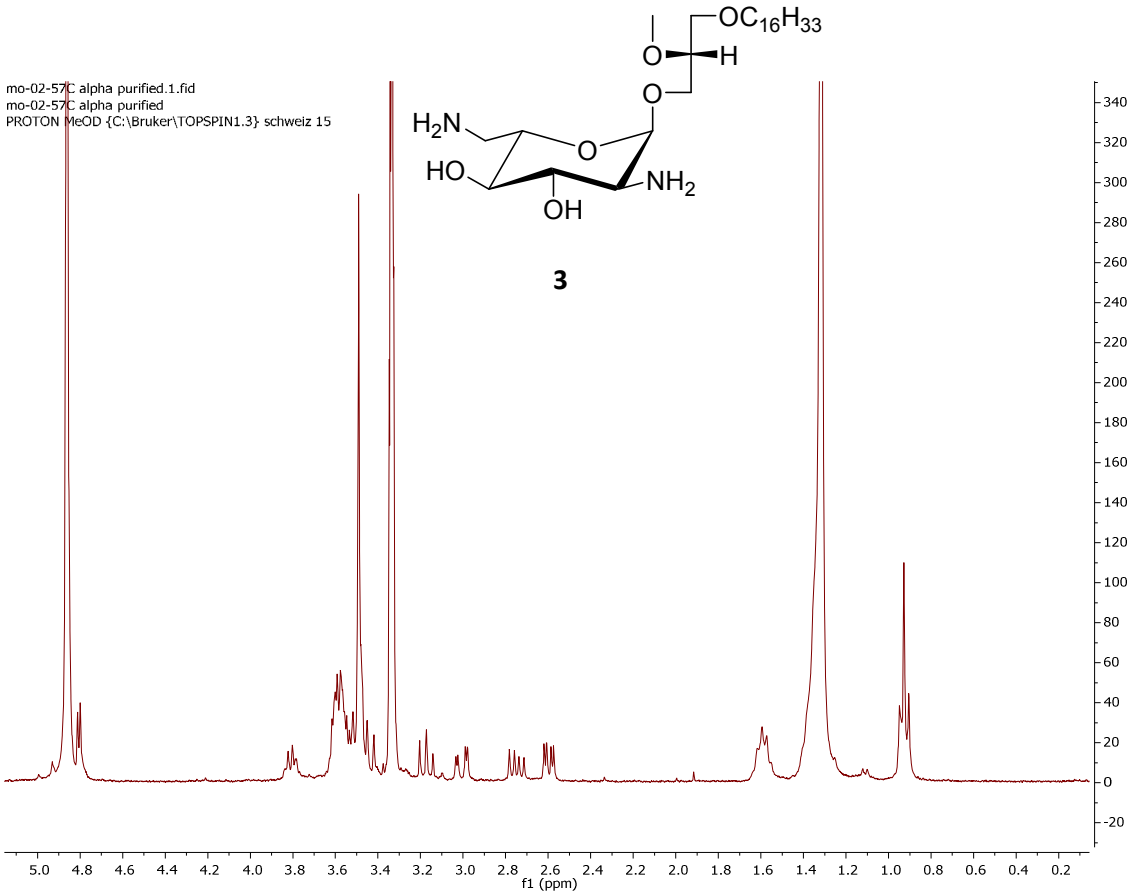
8.2.7 Statistical analysis

The results represent the mean ± standard deviation of 6 independent determinations. Statistical significant difference tests were carried using GraphPadInstat software. The data, that is, the mean values, were subjected to one-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison tests as post hoc test. Comparisons were carried out between the viability of controls and drug-treated cells to determine if statistically significant differences existed between the two groups. The results of the effects of different concentrations of the compounds were also compared for statistically significant differences to determine if the cytotoxic activities of the drugs are dose dependent. The anticancer activities of compounds **1 – 5,** salinomycin, cisplatin and myristylamine tested were also compared using ANOVA followed by Tukey-Kramer multiple comparison tests at the following concentrations: 5, 7.5 and 10 μM to determine if the difference in the potency of the drugs are statistically significant or not. A p value > 0.05 indicates no statistical differences while a p value < 0.001 indicated statistical significant differences.

9. ^{13}C and ^{1}H NMR spectra of compounds 2- 5



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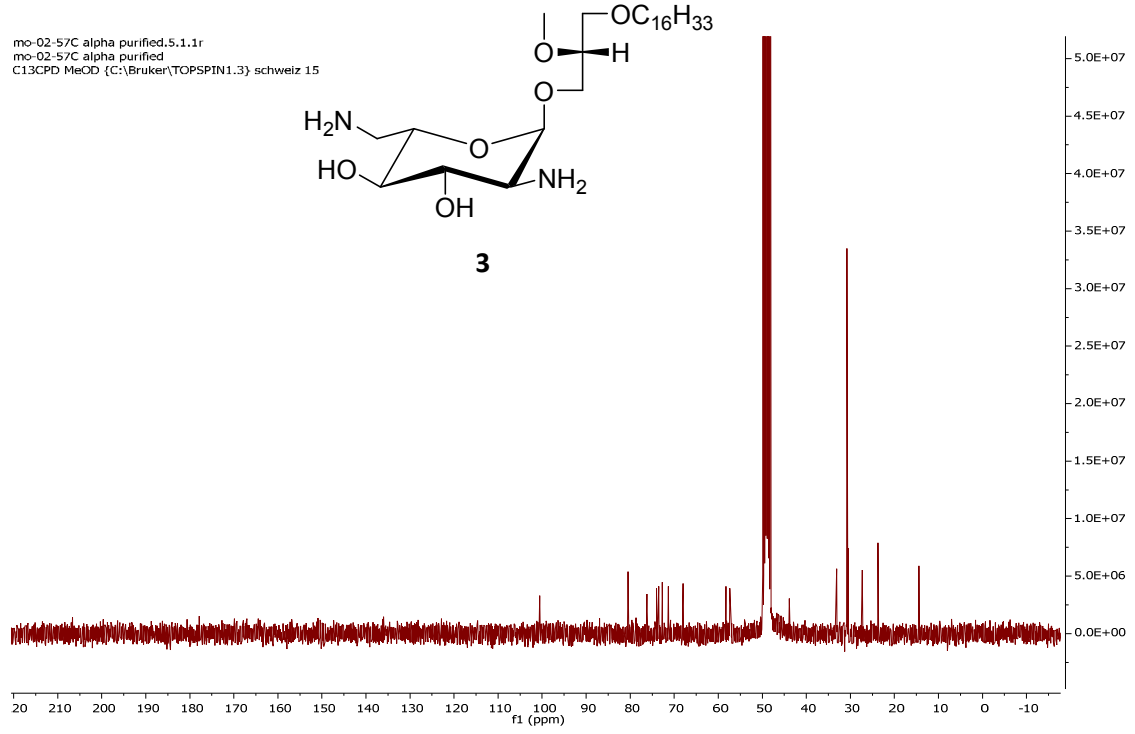
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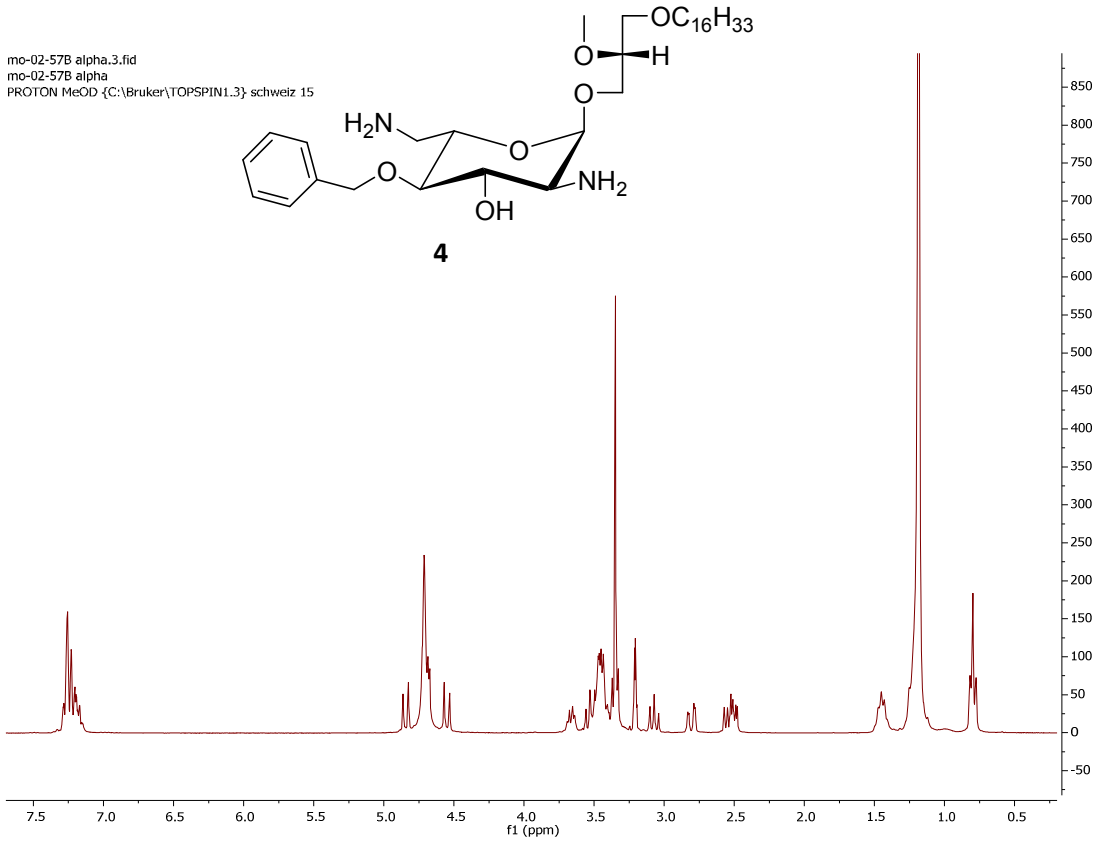
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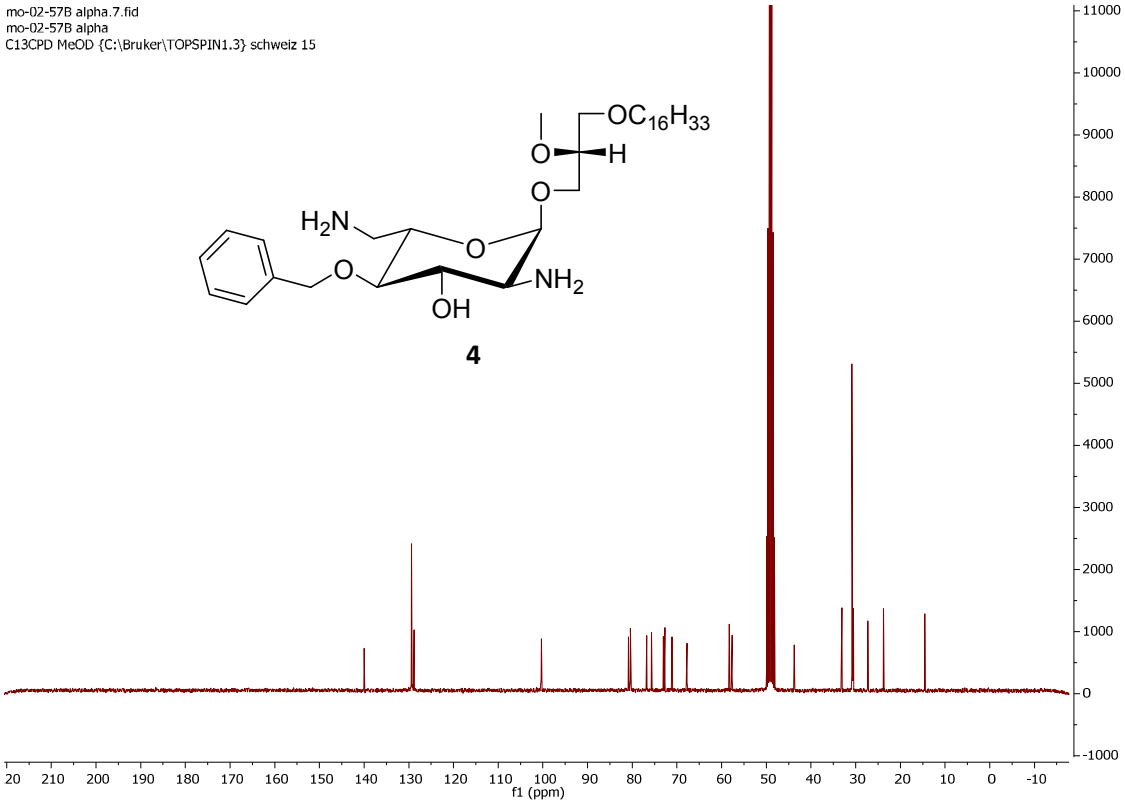
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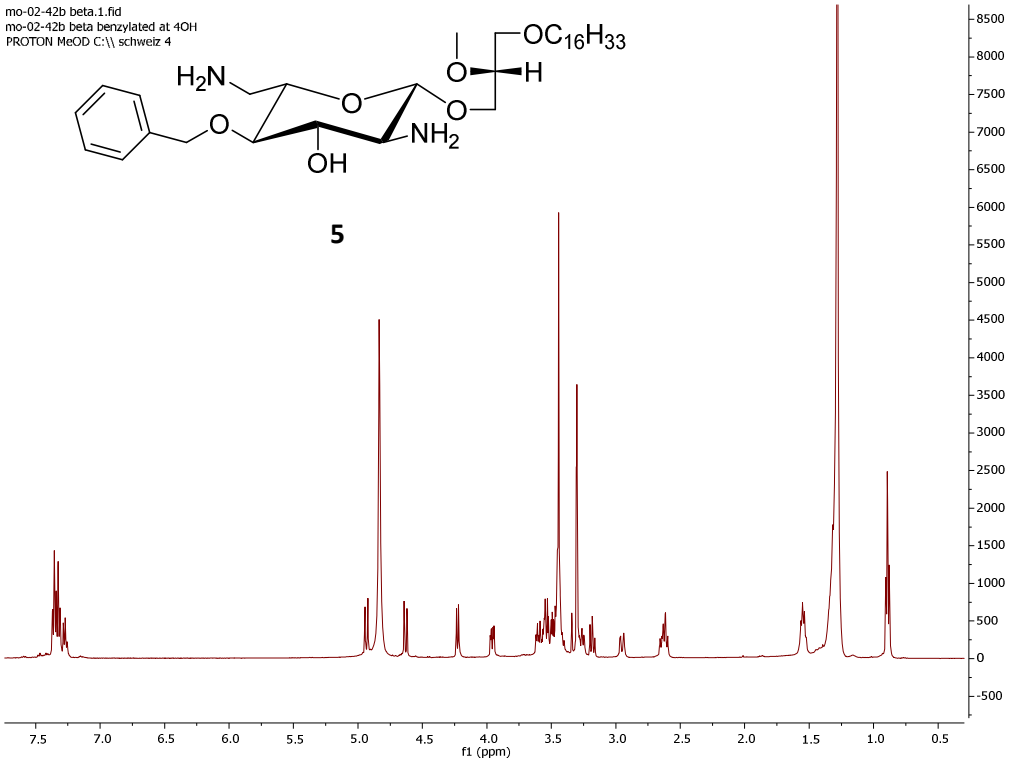
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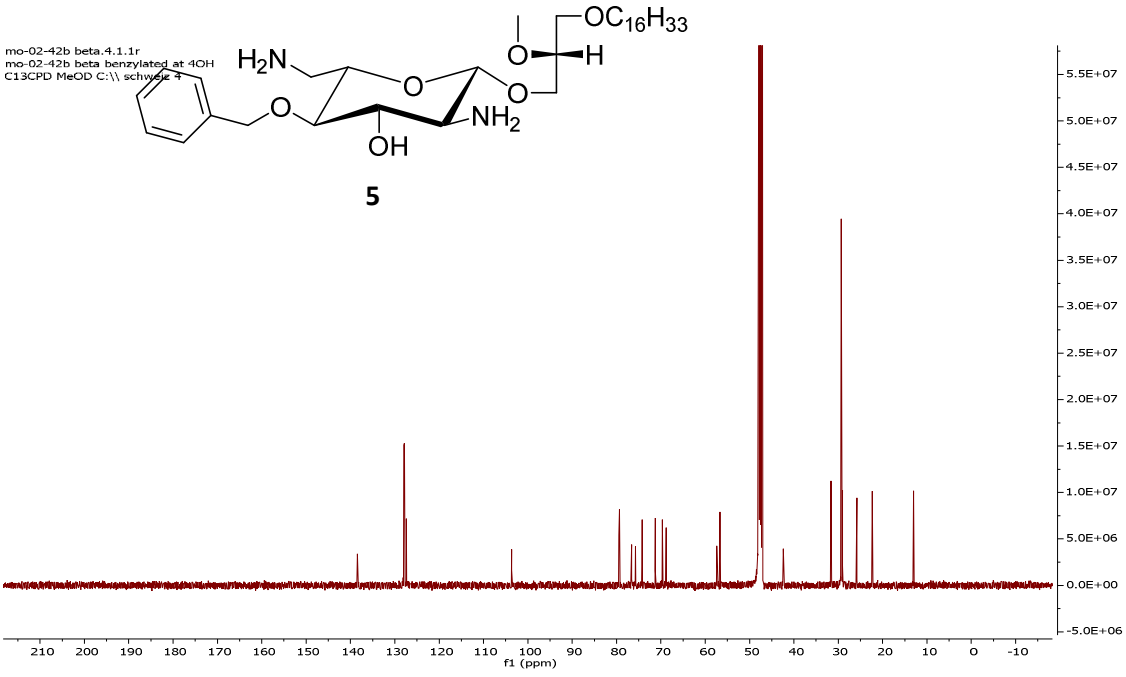
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H
O
O
NH2
OH
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f1 (ppm)

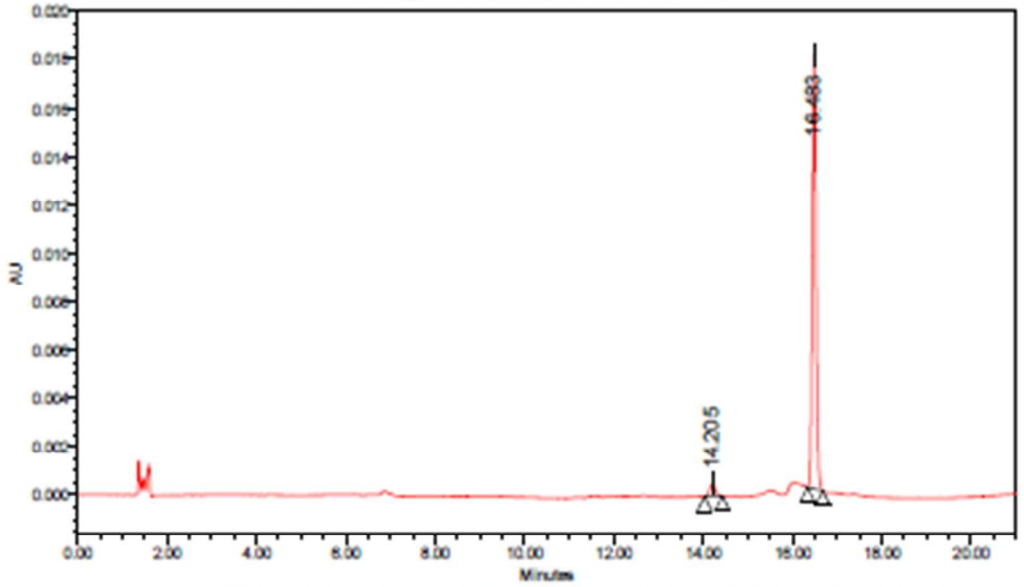
10. Purity report of compounds **4** and **5**

Compound **4** purity report - HPLC-UV chromatogram

Empower 3

Purity report

Chromatogram Overlay with Z Axis Offset



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2	2998 Ch1 271nm@1.2nm	16.483	104102	95.80	17951

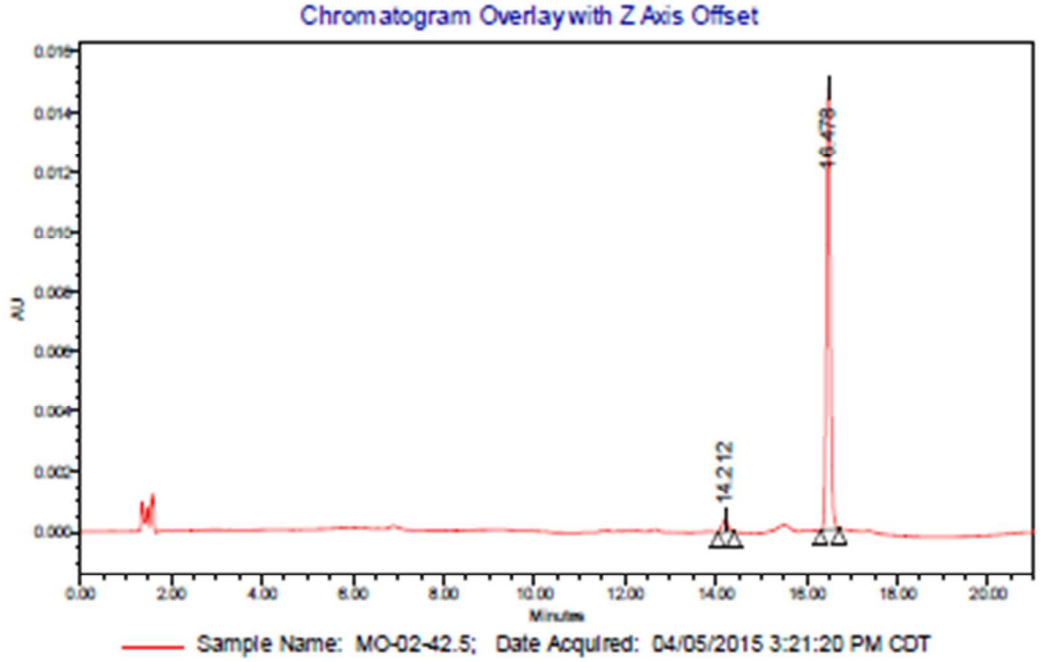
Reported by User: emy komatsu (emyk)
Report Method: Purity report
Report Method ID: 1615
Page: 1 of 1

Project Name: Peak Purity
Date Printed: 04/05/2015
5:10:23 PM US/Central

Compound **5** purity report - HPLC-UV chromatogram

Empower 3

Purity report



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Page: 1 of 1

Project Name: Peak Purity
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