Supporting Information

Aroylhydrazone Glycoconjugate Prochelators Exploit Glucose Transporter 1 (GLUT1) to Target Iron in Cancer Cells

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Contents

	page
Materials and methods	S2
Synthesis and chemical characterization (Figs. S1-S2)	S2
Cell culture and cell-based assays (Figs. S3-S5)	S6
NMR Spectra (Figs. S6-S15)	S10
References	S15

Materials and methods

The preparation of precursors **1**[1], **3**[1], **4**[1], **5**[2], **8**[1] and chelator AH1[3] followed previously reported procedures. 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG)[4] and salicylaldehyde isonicotinoyl hydrazone (SIH)[5] were also prepared as previously reported. Tetrahydrofuran (THF), diethyl ether, *N,N*-dimethylformamide (DMF) and dichloromethane (CH_2Cl_2) were dried by passage through a Vacuum Athmospheres solvent purifier. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, cat# 97062-380, VWR) and propidium iodide (PI, cat# A0402777, Acros Organic) were purchased and used as received. The fluorescein isothiocyanate conjugate of Annexin V (FITC Annexin V, cat# 640906, BioLegend) and Calcein-AM (cat# sc-203865, Santa Cruz Biotechnology) were used per manufacturer's instructions. Deferoxamine mesylate salt (DFO, cat# D9533, Sigma-Aldrich) and all other reagents were obtained commercially and used as received.

Chromatographic purifications on silica gel were conducted on a Biotage Isolera One Flash Chromatography Instrument. HPLC analyses were conducted on an Agilent 1260 Infinity II system using a ZORBAZ Eclipse XDB-C18 (9.4 x 250 mm, 5 μm) column. NMR spectra were recorded on a Bruker Advance-III 400 MHz and a Burker NEO-500 MHz NMR spectrometer at the NMR Spectroscopy Facility of the Department of Chemistry and Biochemistry. The chemical shifts were recorded in ppm relative to tetramethylsilane ($SiMe_4$, δ = 0 ppm) or with the residual solvent resonance as the internal standard. Low- and high-resolution mass spectra (LRMS and HRMS) via electrospray ionization (ESI) methods were obtained at the University of Arizona Analytical & Biological Mass Spectrometry Core Facility. Optical absorption spectra were obtained at ambient temperature using an Agilent 8453 UV-Vis spectrophotometer.

Flow cytometric analyses were performed at the University of Arizona Flow Cytometry Shared Resource (University of Arizona Cancer Center) using a FACSCanto II flow cytometer (BDBiosciences, San Jose, CA) equipped with a 488 nm, air-cooled, 20 mW solid-state laser. List mode data files consisting of 10,000 events gated on FSC (forward scatter) vs SSC (side scatter) were acquired and analyzed using in Diva 8.0 software. (BD Biosciences, San Jose, CA). The detail of data analysis and emission filters for specific dye/fluorophore are mentioned in the experimental section below. Appropriate electronic compensation was adjusted by acquiring cell populations stained with each dye/fluorophore individually, as well as an unstained control.

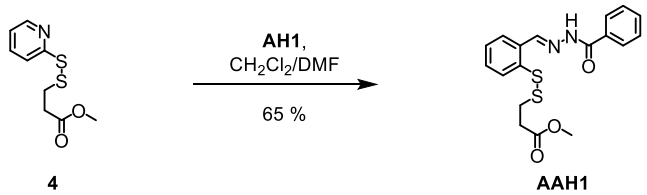
No unexpected or unusually high safety hazards were encountered.

Synthesis and chemical characterization

G6AH1. Pyridyl-4-OTMS glucose **1** (0.24 g, 0.36 mmol) was dissolved in CH_2Cl_2 (3 mL). AH1 (61.6 mg, 0.24 mmol) was dissolved in degassed DMSO (0.2 mL) and added dropwise. The reaction mixture was stirred for 2 h under argon at 25 °C, then the product was extracted using ethyl acetate (20 mL) and washed with water (2 × 5 mL) and brine (2 × 5 mL). The organic phase was dried over sodium sulfate and evaporated under reduced pressure. The crude product was purified by gradient chromatography (100/0 → 80/20, v/v, hexanes/ethyl acetate) to afford the TMS-protected product **2** as a white foam (122 mg, 0.15 mmol, 62% yield). For full deprotection, compound **2** was dissolved in CH_2Cl_2 (0.8 mL), and HCl in diethyl ether (1 N, 0.8 mL) was added while stirring at 0 °C. **G6AH1** precipitated as a white solid, which was collected by centrifugation, washed with cold diethyl ether (2 × 20 mL) and dried under vacuum (73 mg, 0.14 mmol, 91 % yield).^{1}H NMR (500 MHz, CD_3OD) δ 8.98 (s, 1H), 8.15 (m, 1H), 7.97 (m, 2H), 7.82 (m, 1H), 7.61 (m, 1H), 7.53 (m, 2H), 7.48 – 7.36 (m, 2H), 5.07 (d, *J* = 3.7 Hz, 0.53H), 4.47 (d, *J* = 7.8 Hz, 0.45H), 4.43 – 4.32 (m, 1H), 4.25 – 4.12 (m, 1H), 3.97 – 3.64 (m, 1H), 3.46 (m, 0.5H), 3.38 – 3.33 (m, overlap with solvent peak, 1H), 3.30 – 3.25 (m, overlap with solvent peak 1H), 3.13 (m, 0.5H), 2.97 (t, *J* = 7.1 Hz, 2H), 2.76 (t, *J* = 7.1 Hz, 2H); ^{13}C NMR (126 MHz, CD_3OD) δ 173.1, 173.1, 167.1, 147.8, 138.5, 135.4, 134.0, 133.4, 132.3, 131.9, 131.9, 129.7, 129.5, 128.9, 128.9, 98.2, 94.0, 77.9, 76.2, 75.2, 74.7, 73.7, 71.9, 71.7, 70.6, 65.3, 65.2, 34.7, 34.7, 34.1, 34.0. HRMS-ESI *m/z*: $[M+H]^+$ calcd for $C_{23}H_{27}N_2O_8S_2^+$, 523.1203; found 523.1204; $[M+Na]^+$ calcd for $C_{23}H_{26}N_2O_8S_2Na^+$, 545.1023; found 545.1024.

GA2AH1. A solution of pyridyl glucosamine **3** (241 mg, 0.64 mmol) in degassed DMSO (0.8 mL) and acetonitrile (0.4 mL) was heated to 40 °C and a solution of AH1 (82 mg, 0.32 mmol) in degassed DMSO (0.2 mL) and acetonitrile (0.6 mL) was added dropwise. The reaction mixture was allowed to stir under nitrogen for 2 h at 40 °C, then the solvent was evaporated under reduced pressure. The crude product was purified by gradient chromatography (100/0 → 80/20, v/v, MeOH/CH_2Cl_2). The resulting viscous colorless oil was dried under high vacuum to afford **GA2AH1** as a white foam

(110 mg, 0.21 mmol, 66% yield).[1]H NMR (500 MHz, CD_3OD) δ 9.00 (s, 1H), 8.16 (m, 1H), 8.02 – 7.94 (m, 2H), 7.88 – 7.85 (m, 1H), 7.64 – 7.59 (m, 1H), 7.57 – 7.51 (m, 2H), 7.49 – 7.38 (m, 2H), 5.10 (d, *J* = 3.5 Hz, 0.8H), 4.58 (d, *J* = 8.4 Hz, 0.2H), 3.88 – 3.83 (m, 1H), 3.82 – 3.77 (m, 2H), 3.72 – 3.59 (m, 2H), 3.44 – 3.33 (m, 1H), 3.04 – 2.98 (m, 2H), 2.73 – 2.66 (m, 2H); [13]C NMR (126 MHz, CD_3OD) δ 173.8, 167.1, 148.1, 138.7, 135.5, 134.0, 133.4, 132.7, 131.9, 129.8, 129.5, 129.0, 128.9, 92.6, 73.1, 72.6, 72.5, 62.8, 55.9, 36.6, 35.0. HRMS-ESI *m/z*: $[M+H]^+$ calcd for $C_{23}H_{28}N_3O_7S_2^+$, 522.1363; found 522.1365; $[M+Na]^+$ calcd for $C_{23}H_{27}N_3O_7S_2Na^+$, 544.1183; found 544.1183.



Scheme S1. Synhtesis of **AAH1**.

AAH1. 3-(2-pyridyldithio)propanoate **4** (190 mg, 0.83 mmol) was dissolved in degassed CH_2Cl_2 (3.5 mL) and cooled to 0 °C. AH1 (70.8 mg, 0.27 mmol) was dissolved in degassed CH_2Cl_2 (2 mL plus minimal degassed DMF to assist the solubilization), then added dropwise to the reaction mixture at 0 °C and stirred under nitrogen for 4 h. The solvent was removed, and the crude was dissolved in ethyl acetate (15 mL) and extracted with deionized water (5 mL). After evaporation of the organic phase, the residue was purified by gradient column chromatography (100/0 → 80/20, v/v, hexanes/ethyl acetate) and isolated as a solid (67 mg, 0.18 mmol, 65% yield). [1]H NMR (400 MHz, $CDCl_3$) δ 11.03 (s, 1H), 8.93 (s, 1H), 8.01 (d, *J* = 7.6 Hz, 2H), 7.93 (d, *J* = 7.7 Hz, 1H), 7.65 (d, *J* = 7.9 Hz, 1H), 7.47 (m, 1H), 7.38 (m, 2H), 7.28 (m, 1H), 7.16 (m, 1H), 3.62 (s, 3H), 2.83 (t, *J* = 7.1 Hz, 2H), 2.64 (t, *J* = 7.1 Hz, 2H); [13]C NMR (101 MHz, $CDCl_3$) δ 172.4, 164.7, 146.2, 136.7, 134.0, 133.1, 132.0, 131.1, 130.5, 128.6, 128.3, 128.0, 127.9, 52.1, 33.9, 32.9. HRMS-ESI *m/z*: $[M+H]^+$ calcd for $C_{18}H_{19}N_2O_3S_2^+$, 375.0831; found 375.0829.

1,3,5-tri-O-tert-butyldimethylsilyl-6-O-trityl-2-deoxy-D-glucopyranose 6. *Tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMS triflate, 3.4 mL, 14.6 mmol) was added dropwise to a suspension of 6-O-Trityl-2-deoxy-D-glucopyranose **5** (1.32 g, 3.25 mmol) in dry CH_2Cl_2 at 0 °C and stirred for 1 h. The reaction mixture was poured into a saturated $NaHCO_3$ solution (20 mL) and extracted with ethyl acetate (3 × 10 mL). Purification by gradient chromatography (100/0 → 90/10, v/v, hexane/ethyl acetate) afforded the anomeric mixture of **6** (2.31 g, 3.09 mmol, 95% yield). Interestingly, the beta form crystallized upon standing at room temperature overnight. [1]H NMR (400 MHz, $CDCl_3$) δ 7.53 – 7.21 (m, Ar, 15H), 5.38 (br s, 0.7H), 4.92 (dd, J= 9.4, 2.1 Hz, 0.3H), 4.11 – 4.05 (m, 1H), 3.75 – 3.54 (m, 1H), 3.45 – 3.37 (m, 1H), 3.25 – 3.09 (m, 2H), 2.17 – 2.03 (m, 1H), 1.73 – 1.65 (m, 1H), 1.01 – -0.37 (m, 45H). Crystallized beta anomer: [1]H NMR (400 MHz, $CDCl_3$) [1]H NMR (400 MHz, $CDCl_3$) δ 7.51 – 7.46 (m, 6H), 7.29 – 7.17 (m, 9H), 4.89 (dd, *J* = 9.4, 2.1 Hz, 1H), 3.66 (m, 1H), 3.53 (td, *J* = 9.2, 1.9 Hz, 1H), 3.34 (dd, *J* = 9.3, 2.0 Hz, 1H), 3.16 – 3.08 (m, 2H), 2.10 (m, 1H), 1.63 (m, 1H), 0.95 (s, 9H), 0.89 (s, 9H), 0.61 (s, 9H), 0.25 (s, 3H), 0.23 (s, 3H), 0.09 (s, 3H), 0.06 (s, 3H), -0.04 (s, 3H), -0.41 (s, 3H); [13]C NMR (126 MHz, $CDCl_3$) δ 144.6, 129.1, 128.0, 127.0, 94.6, 86.5, 76.8, 73.8, 73.6, 64.9, 43.7, 26.4, 26.0, 25.9, 18.4, 18.2, 17.7, -2.9, -2.9, -4.0, -4.1, -4.5, -5.4. LRMS-ESI *m/z*: $[M+Na]^+$ calcd for $C_{43}H_{68}O_5Si_3Na^+$, 771.43; found 771.45.

1,3,5-tri-O-tert-butyldimethylsilyl-2-deoxy-D-glucopyranose 7. BCl_3 (1.57 mL, 1.57 mmol, 1.0 M in CH_2Cl_2) was added dropwise via syringe to a solution of **6** (1.96 g, 2.62 mmol) in CH_2Cl_2 (13 mL) at -40 °C.[6] After 5 min, the reaction was quenched by the addition of anhydrous MeOH. The solution was poured into a saturated $NaHCO_3$ solution (10 mL), stirred for 5 min and then extracted with CH_2Cl_2 (2 × 15 mL). The organic phase was washed with brine (2 × 10 mL), dried over $MgSO_4$ and concentrated in vacuo. The residual oil was purified by gradient column chromatography (100/0 → 90/10, v/v, hexane/ethyl acetate) to afford **7** as an anomeric mixture (0.82 g, 1.62 mmol, 62% yield). [1]H NMR (500 MHz, $CDCl_3$) δ 5.24 – 4.81(m, 1H), 4.08 – 3.61 (m, 4H), 3.42 – 3.37 (m, 1H), 2.02 – 1.56 (m, 3H), 0.94 – 0.87 (m, 27H), 0.13 – 0.07 (m, 18H). Alpha form: [1]H NMR (500 MHz, $CDCl_3$) δ 5.24 (br s, 1H), 4.04 (m, 1H), 3.77 – 3.63 (m, 3H), 3.43 – 3.37 (m, 1H), 1.99 (m, 1H), 1.85 – 1.81 (m, 1H), 1.66 – 1.61 (m, 1H), 0.91 (s, 9H), 0.90 (s, 9H), 0.90 (s, 9H), 0.11 – 0.08 (m, 18H). [13]C NMR (126 MHz, $CDCl_3$) δ 92.3, 73.4, 73.4, 70.6, 62.3, 41.7, 26.4, 26.2, 25.7, 18.4, 18.2, 18.0, -2.9, -2.9, -4.3, -4.4, -4.7. LRMS-ESI *m/z*: $[M+Na]^+$ calcd for $C_{24}H_{54}O_5Si_3Na^+$, 529.32; found 529.34.

1,3,5-tri-O-tert-butyldimethylsilyl-2-deoxy-D-glucopyranose-3-(2-pyridyldithio)propanoate 9. Compound **7** (0.84 g, 1.65 mmol), 3-(2-pyridyldithio)propionic acid **8** (0.39 g, 1.82 mmol), N,N′-dicyclohexylcarbodiimide (DCC, 0.30 g, 1.44 mmol) and 4-dimethylaminopyridine (DMAP, 17.6 mg, 0.14 mmol) were combined in CH_2Cl_2 (12 mL) and allowed to stir at 25 °C for 15 h. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and filtered through a celite plug. The resulting solution was concentrated under vacuum and purified by gradient column (100/0 → 90/10, v/v, hexane/ethyl acetate) to afford **9** as an anomeric mixture (0.75 g, 1.07 mmol, 65% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 – 8.44 (m, 1H), 7.72 – 7.61 (m, 2H), 7.11 – 7.06 (m, 1H), 5.25 – 4.73 (m, 1H), 4.53 – 4.35 (m, 1H), 4.17 – 3.99 (m, 2H), 3.86 – 3.62 (m, 1H), 3.38 – 3.32 (m, 1H), 3.07 – 3.02 (m, 2H), 2.82 – 2.75 (m, 2H), 2.14 – 1.96 (m, 1H), 1.69 – 1.56 (m, 1H), 0.93 – 0.84 (m, 27H), 0.12 – 0.02 (m, 18H). Alpha form: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.46 (m, 1H), 7.71 – 7.61 (m, 2H), 7.08 (m, 1H), 5.21 (br s, 1H), 4.38 (dd, *J* = 11.6, 2.4 Hz, 1H), 4.09 (m, 1H), 4.02 (m, 1H), 3.81 (m, 1H), 3.38 (m, 1H), 3.03 (t, *J* = 7.4 Hz, 2H), 2.78 (m, 2H), 1.99 (m, 1H), 1.63 (m, 1H), 0.90 (s, 9H), 0.88 (s, 9H), 0.88 (s, 9H), 0.11 – 0.03 (m, 18H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.8, 160.1, 150.0, 137.3, 120.9, 119.9, 92.1, 73.5, 71.4, 70.7, 64.6, 41.4, 33.9, 33.3, 26.4, 26.1, 25.6, 18.4, 18.1, 18.0, -2.9, -2.9, -4.3, -4.4, -4.7. LRMS-ESI *m/z*: $[M+H]^+$ calcd for $C_{32}H_{62}NO_6S_2Si_3^+$, 704.33; found 704.30.

2-deoxy-D-glucopyranose-3-(2-pyridyldithio)propanoate 10. Compound **9** (448.6 mg, 0.64 mmol) was dissolved in dry THF (6.4 mL) and cooled to 0 °C. Hydrogen fluoride pyridine complex (HF/pyridine 70%/30%, 2.64 mL, 101.92 mmol, 160 equiv.) was added to the reaction mixture and stirred for 1 h at 0 °C. The reaction was neutralized by saturated $NaHCO_3$, and the aqueous layer was extracted with ethyl acetate (10 mL × 3). The organic layers were combined, dried over Na_2SO_4, and the solvent was evaporated. The crude residue was purified by gradient column (100/0 → 80/20, v/v, CH_2Cl_2 /MeOH) to obtain **10** as a white foam (137 mg, 0.38 mmol, 60% yield).^{1}H NMR (400 MHz, CD_3OD) δ 8.43 – 8.37 (m, 1H), 7.87 – 7.79 (m, 2H), 7.25 – 7.20 (m, 1H), 5.24 (br s, 0.6H), 4.78 (dd, *J* = 9.7, 2.0 Hz, 0.4H), 4.45 – 4.34 (m, 1H), 4.29 – 4.17 (m, 1H), 3.96 – 3.85 (m, 1H), 3.61 – 3.37 (m, 1H), 3.27 – 3.14 (m, 1H), 3.07 (t, *J* = 6.9 Hz, 2H), 2.77 (t, *J* = 6.9 Hz, 2H), 2.18 – 2.01 (m, 1H), 1.63 – 1.43 (m, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.1, 173.0, 161.1, 150.4, 139.2, 139.2, 122.5, 121.2, 115.7, 95.2, 92.8, 75.4, 73.5, 72.9, 72.4, 71.2, 69.5, 65.5, 65.4, 41.8, 39.5, 34.7, 34.6. HRMS-ESI *m/z*: $[M+H]^+$ calcd for $C_{14}H_{20}NO_6S_2^+$, 362.0727; found 362.0725.

2DG6AH1. Compound **10** (100 mg, 0.27 mmol) was suspended in degassed CH_2Cl_2 (1.5 mL). AH1 (34.6 mg, 0.135 mmol) was dissolved in degassed CH_2Cl_2 (0.5 mL) with minimal DMSO (2 drops) and added to the reaction mixture. The reaction was stirred under a nitrogen atmosphere for 4 h at 25 °C, then the solvent was evaporated and the residue was initially purified by gradient column chromatography on silica gel (100/0 → 80/20, v/v, CH_2Cl_2 /MeOH). Further purification by preparative HPLC (95/5 → 5/95, v/v, acetonitrile/H_2O) afforded **2DG6AH1** as a white foam (46 mg, 0.09 mmol, 63% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.98 (s, 1H), 8.16 (m, 1H), 7.99 – 7.93 (m, 2H), 7.83 (m, 1H), 7.64 – 7.57 (m, 1H), 7.56 – 7.50 (m, 2H), 7.49 – 7.37 (m, 2H), 5.22 (d, *J* = 3.4 Hz, 0.6 H), 4.77 (dd, *J* = 9.7, 2.0 Hz, 0.4H), 4.42 – 4.33 (m, 1H), 4.25 – 4.16 (m, 1H), 3.94 – 3.84 (m, 1H), 3.60 – 3.36 (m, 1H), 3.27 – 3.12 (m, 1H), 2.99 (t, *J* = 7.0 Hz, 2H), 2.78 (t, *J* = 7.0 Hz, 2H), 2.17 – 2.00 (m, 1H), 1.62 – 1.42 (m, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.2, 173.1, 167.1, 147.8, 138.5, 135.4, 134.1, 133.4, 132.3, 131.9, 131.9, 129.8, 129.5, 129.0, 128.9, 95.2, 92.8, 75.4, 73.5, 73.0, 72.4, 71.2, 69.5, 65.4 (br, merged peaks), 41.8, 39.5, 34.7, 34.7, 34.1, 34.1. HRMS-ESI *m/z*: $[M+Na]^+$ calcd for $C_{23}H_{26}N_2O_7S_2Na^+$, 529.1074; found 529.1078.

HPLC analysis of glycoconjugates and aglycone. Prior to biological testing, the purity of the prepared compounds was assessed by HPLC on an Agilent 1260 Infinity II system equipped with a ZORBAZ Eclipse XDB-C18 (9.4 x 250 mm, 5 μm) column. The mobile phase consisted of water/acetonitrile 95/5 (A) and water/acetonitrile 5/95 (B), set as follows: 0.00 min 100% A → 12.0 min 100% B → 14.0 min 100% B → 16.0 min 100% A → 18.0 min 100% A; at a rate of 1.0 mL/min for 18 min with 20 μL per injection. The glycoconjugate compounds eluted as anomeric mixtures (i.e., two sharp overlapping peaks, Figure S1) as confirmed by ^{1}H NMR data (Figures S6-S15). Conversely, the aglycone AAH1 eluted as a single peak.

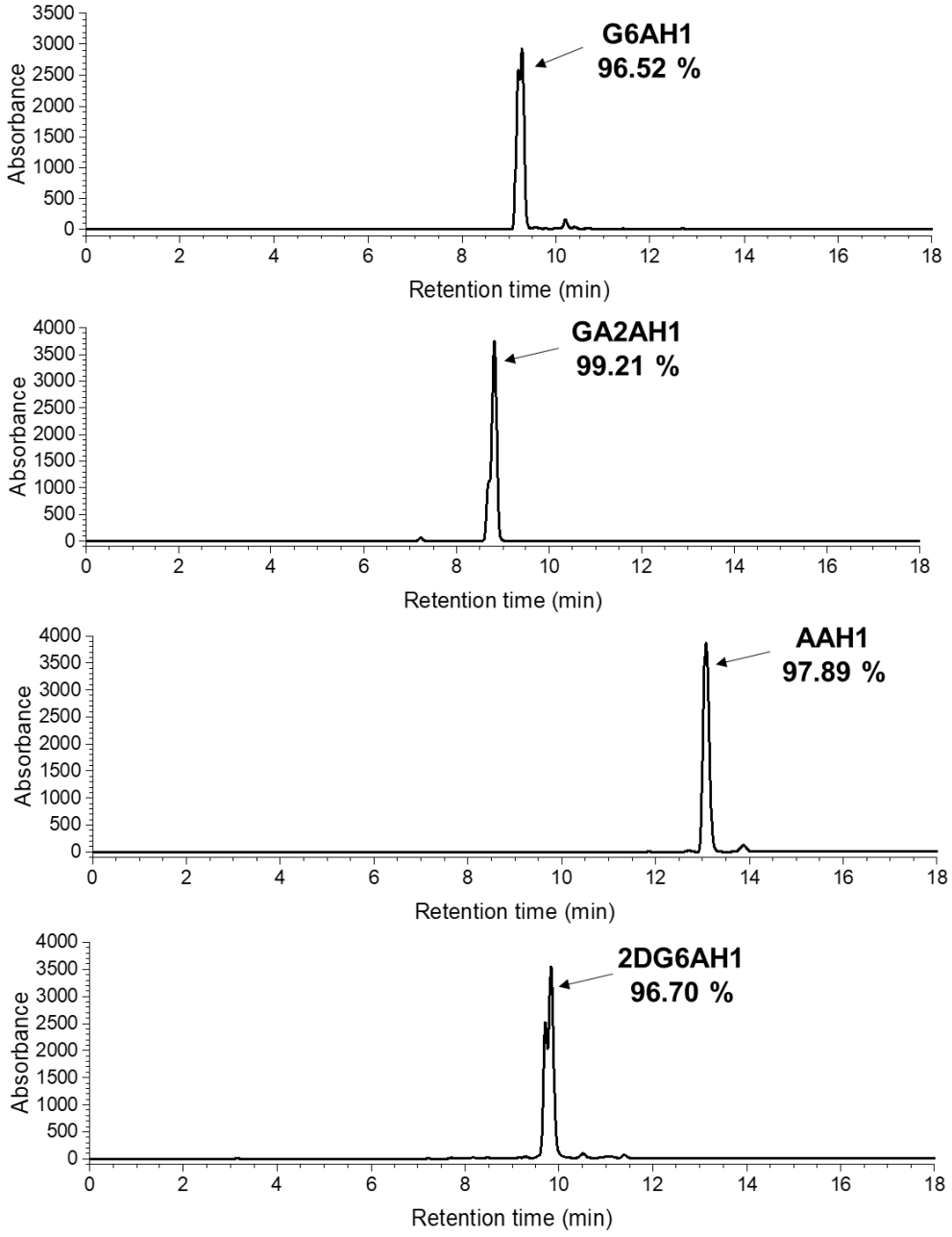


Figure S1. HPLC chromatograms and purity of isolated **G6AH1**, **GA2AH1**, **AAH1**, and **2DG6AH1**.

Stability in growth media. Stock solutions were prepared in DMSO and diluted in phenol-red-free EMEM supplemented with 10% FBS as in the media for cell culture. The final concentration of the tested compounds is 50 µM. A mixture containing an identical amount of DMSO (0.1% v/v) was used to obtain blank spectra. The compounds stability was assessed over 24 h by monitoring the intensity decrease of λ_{max} absorption band at 305 nm.

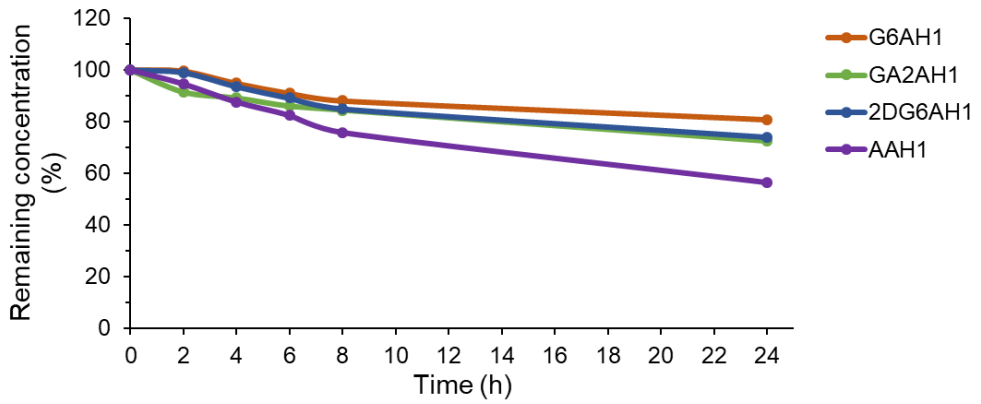


Figure S2. The tested compounds were added to phenol-red-free EMEM supplemented with 10% FBS to a final concentration of 50 µM (0.1% DMSO v/v). The absorbance of the compounds was monitored at 305 nm. The stability after 8 h was as follows: G6AH1 88%, GA2AH1 84%, 2DG6AH1 85%, AAH1 76%; and after 24 h: G6AH1 81%, GA2AH1 72%, 2DG6AH1 74%, AAH1 58%.

Cell culture and cell-based assays

The A2780 (Fox Chase Cancer Center, 03-26) cells were grown in RPMI medium (Corning® RPMI 1640) supplemented with 10% fetal bovine serum, penicillin (100 I.U./mL) and streptomycin (100 µg/mL). The MDA-MB-231 (ATCC® HTB-26™) and MRC-5 (ATCC® CCL-171™) cell lines were maintained in Eagle's Minimum Essential Medium (Corning® 500 mL MEM) supplemented with 10% fetal bovine serum, penicillin (100 I.U./mL) and streptomycin (100 µg/mL). All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Phenol red-free RPMI-1640 medium was purchased from Quality Biological.

GLUT1 and Turbo-635 transduced MDA-MB-231 and MRC-5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic mixture (Gibco®, final concentrations: 100 units/mL penicillin G sodium, 100 ug/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B), and puromycin (2.5 µg/mL, Thermo Fisher Scientific).

Cytotoxicity assays

MTT viability assays were conducted by standard methods. Cells were seeded in growth media (100 µL) with the following cell density per well in 96-well plates: A2780, 2000 cells/well; MDA-MB-231, 2000 cells/well; MRC-5, 5000 cells/well, and allowed to attach for at least 24 h. Test compounds dissolved in DMSO were diluted in growth media to the concentration range from 100 µM to 0.16 µM (with final DMSO concentration limited to 0.1%). Cells were incubated in the presence of the test compounds (200 µL in growth media) for 72 h. After removing the media containing the test compounds, the MTT solution (prepared in media without FBS, 0.5 mg/mL) was added to each well (100 µL) and incubated for 4 h. Following media removal, DMSO (200 µL) was added to each well to dissolve the purple formazan crystals and the plates were incubated at 37 °C for 30 minutes. The absorbance of the solutions at 570 nm was recorded on a BioTek Synergy™ 2 microplate reader and data were analyzed using sigmoidal fits (DoseResp function, Origin software) to obtain IC_{50} values. The reported IC_{50} values are the average of at least three independent experiments, and values are given as mean plus/minus standard deviation.

The cytotoxicity assays in the presence of GLUT1 inhibitor phloretin followed a similar procedure: A2780 cells were treated with a serial dilution of the tested compounds ranging from 100 to 0.2 µM prepared in phloretin-containing RPMI medium (12.5 µM). The toxicity profiles of the compounds were then evaluated by MTT assay as described above.

Toxicity assays of MDA-MB-231 and MRC-5 transduced cell lines (GLUT1 and Turbo-635) were conducted exactly as for parental cell lines, and puromycin dihydrochloride (Thermo Fisher Scientific) was not added in growth media for MTT assay.

Apoptosis analysis by flow cytometry

A2780 cells were seeded at a density of 2×10^5 cells per well in 6-well plates and cultured for 24 h at 37 °C. The culture media were replaced with fresh media containing the test compounds (20 µM) and incubated for 48 h. Cells were then harvested (including detached and attached cells) and washed once with cold DPBS and annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). The cell number was determined, and cell density was adjusted to 1×10^6 cells/mL. Annexin V-FITC conjugate (5 µL) and propidium iodide (PI) solutions (final concentration 1 µg/mL) were added to the cell suspensions (100 µL). The cells were stained at room temperature for 15 min, then annexin-binding buffer (400 µL) was added and mixed gently. The prepared samples were kept on the ice and analyzed by a flow cytometer within 1 hour with excitation at 488 nm and emission at 530/30 nm and 585nm/42 nm for FITC and PI, respectively. Appropriate electronic compensation was adjusted by acquiring cell populations stained with Annexin V-FITC/PI individually. For analysis, the cells were grouped in four quadrants as follows: normal (Q1, Annexin V-FITC/PI -/-), early apoptosis (Q2, Annexin V-FITC/PI +/-), late apoptosis (Q3, Annexin V-FITC/PI +/+), and necrosis (Q4, Annexin V-FITC/PI -/+).

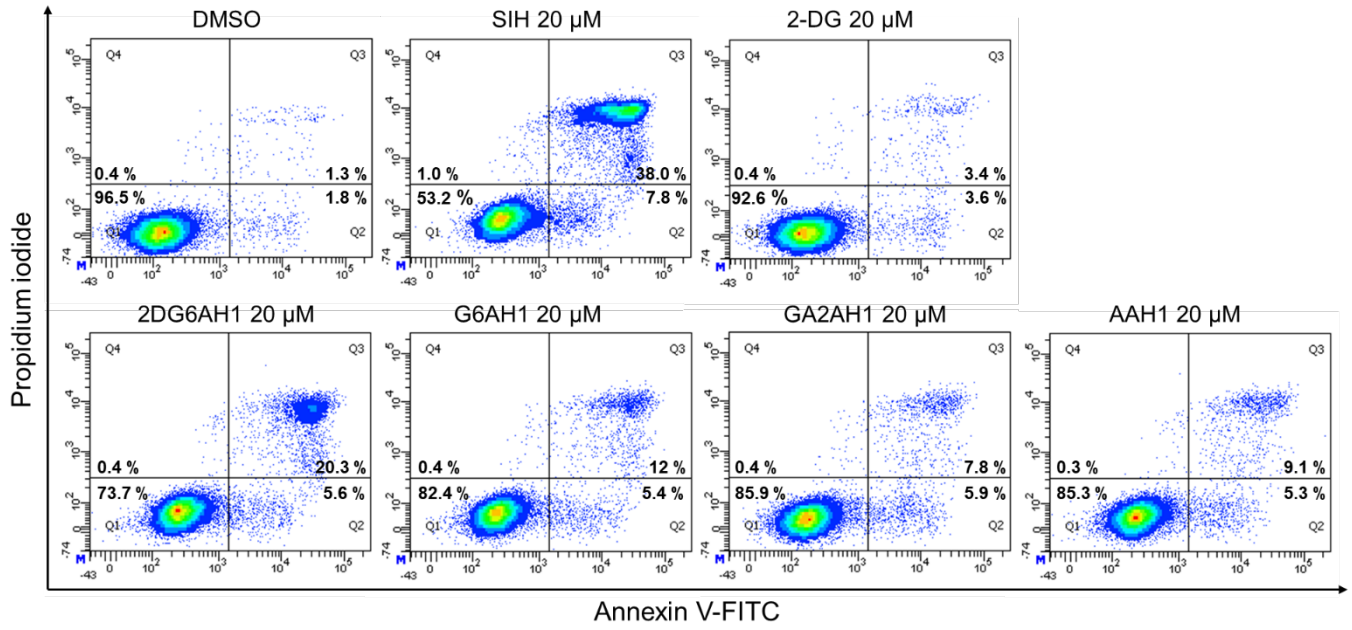


Figure S3. Apoptosis induced by AH1 glycoconjugates in human ovarian (A2780) cancer cells.

Intracellular iron-binding assay

A2780 cells were seeded at a density of 1×10^4 cells per well in 96-well plates and cultured for 24 h at 37 °C. The cells were washed gently with DPBS twice to remove residual FBS, and incubated with calcein-AM (0.1 µM) prepared in serum-free RPMI-1640 medium (without phenol red) for 15 min at 37 °C. The cells were then washed with DPBS to remove residual calcein-AM and fresh medium was added to each well. After obtaining baseline readings of calcein fluorescence for each well, the test compounds were added in RPMI medium (20 µM, serum-free and no phenol red) and the plate was read after 20 min and 1 h. The calculation of fluorescence change (%) was conducted by comparing readings before and after the loading of test compounds (or vehicle control). Data are reported as averages from three different experiments plus/minus one standard deviation.

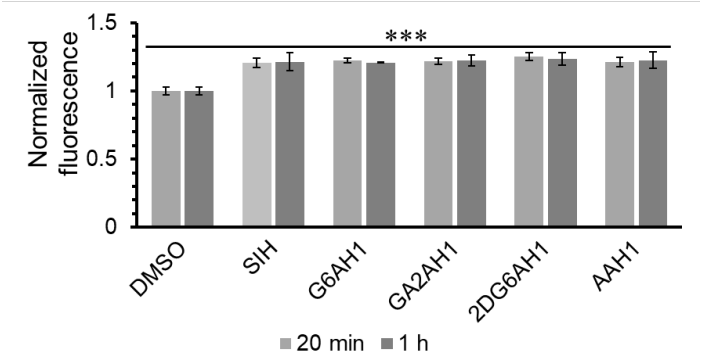


Figure S4. Qualitative intracellular iron binding measured through the calcein assay in A2780 cells. Cell cultures were incubated with calcein-AM (0.1 μM, 15 min), washed, and then incubated with the test compounds (20 μM, 20 min or 1 h). Experiments were conducted in triplicate and the values shown are averages ± standard deviation. *** $p < 0.001$.

Competition assay of transporter-mediated uptake

A2780 cells were plated at 2×10^5 cells per well in 6-well plates and allowed to adhere for at least 24 h. The growth media were then removed and the cells were incubated for 12 h in glucose-free RPMI. Cells were then treated with prepared RPMI containing 1 g/L glucose (unstained control), glucose-containing RPMI with 2-NBDG (100 μM) (stained control), or a combination of 2-NBDG (100 μM) and test compounds (50 μM) for 20 minutes. As a positive control, cells were treated with glucose-transporter inhibitor phloretin (50 μM) and 2-NBDG (100 μM) in the same conditions. Next, the cells were washed with DPBS (1 mL) and detached by addition of 0.25% Trypsin-EDTA (200 μL) and incubation for 2 minutes at 37 °C. Following the addition of RPMI (1 mL), the cell suspensions were centrifuged at 125 g for 7 minutes. The resulting pellets were suspended in DPBS (0.5 mL), transferred to a flow cytometry tube, stored on ice, and analyzed by flow cytometry within 1 hour. The 2-NBDG probe was excited at 488 nm and the fluorescence was recorded through a 530nm/30nm and band-pass filter in the FL1 channel. Data are obtained as the average of three sets of geometric means of the flow cytometry histogram and plotted as the percent difference from the control plus/minus the standard deviation.

Cell viability assays in the presence of phloretin

The toxicity of phloretin was evaluated in serial dilutions ranging from 100 to 0.2 μM by MTT assays. For viability tests of AH1 glycoconjugates in the presence of phloretin, we chose a phloretin concentration of 12.5 μM, which did not affect the proliferation of A2780 cells over a 72 h period. This experiment was conducted in biological triplicate.

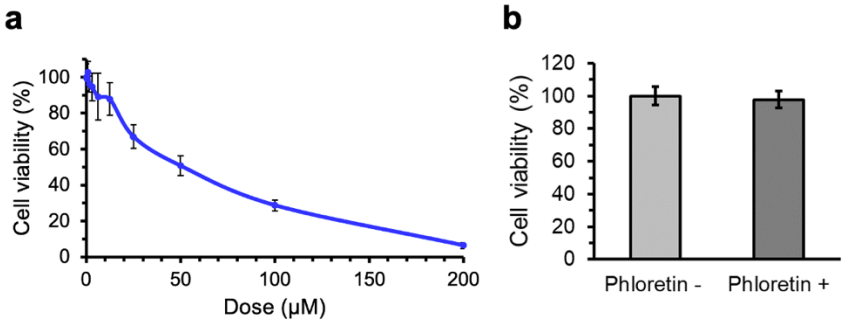


Figure S5. (**a**) Viability plot of phloretin (200–0.39 μM) in A2780 cells after 72 h incubations. (**b**) Single-dose response to phloretin (12.5 μM) and untreated control in A2780 cells over a period of 72 h (n=3).

Western blot

For analysis of GLUT1 and β-actin levels, A2780, MDA-MB-231, and MRC-5 cells were cultured for 48 h and then suspended in lysis buffer (64 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) supplemented with Halt protease inhibitor cocktail (Thermo Fisher Scientific Cat# 78430) on ice, and incubated at 100 °C for 5 min. Then, extracts were sonicated (10% amplitude, 30 s) on ice. Protein determination in supernatants was conducted using a bicinchoninic acid-based protein assay (Thermo Fisher Scientific). Whole-cell lysates (30 μg) were loaded onto 12% SDS-polyacrylamide gels and separated by electrophoresis (165 V, 40 min). Prestained protein ladder (10–250 kDa, cat# 83-660, Prometheus Protein Biology Products) was used as a comparison. Proteins were transferred to ploy vinylidene difluoride membranes (PVDF 40 V, 1h) and blocked for 1 h at 21 °C in 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween-20 (TBST). After washing with TBST 3 times, membranes were incubated overnight at 4 °C with the following primary antibodies: mouse mAb anti-GLUT1 IgG1 (1:500, clone A-4, sc- 377228; Santa Cruz Biotechnology) in 5% BSA in TBST and mouse monoclonal anti-β-actin (1:5000, clone AC-15, A1978; Sigma-Aldrich) in 5% milk in TBST. On the following day after washing with TBST 3 times, the membranes were incubated with m-IgG$_K$ HRP-labeled secondary antibody (1:5000, sc-516102; Santa Cruz Biotechnology) in TBST for 1 h at 21 °C. After incubation with secondary antibodies, membranes were washed with TBST 3 times and incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific). Chemiluminescence signals were captured and analyzed by using UVP ChemoStudio™ touch, 815 (Analytik Jena, USA).

Plasmids preparation

Viral vectors for GLUT1 overexpression and control Turbo-635 were constructed by Gibson Assembly. These plasmids were designed to put the expression our genes of interest (GLUT1 or Turbo-635) and the selection marker (puromycin resistance gene) under the control of a single cytomegalovirus (CMV) promoter. To do this, the constructs were designed to concatenate the various fragments in the following order: a CMV promoter followed by the open reading frame (ORF) of either GLUT1 or Turbo-635, followed by an internal ribosome entry site (IRES) and a puromycin resistance gene. We employed the control Turbo-635 vector instead of an empty vector so that the downstream IRES-regulated antibiotic resistance gene is expressed at a similar level as for the GLUT1 vector. The sources of the various DNA fragments used were as follows: the lentivirus backbone used was derived from pLenti6V5 (Thermo Fisher); the CMV promoter was derived from pIRESPuro3 (Clonetech); the internal ribosome entry site followed by the puromycin resistance gene (IRES-Puro) was derived from pIRESPuro3 (Clonetech); the GLUT1 and Turbo-635 ORFs were synthesized as gBlocks from Integrated DNA Technologies (IDT).

Production of lentiviral particles and viral transduction

The ViraPower Lentiviral expression system was used to produce lentivirus for ectopic expression of GLUT1 or Turbo-635 according to the manufacturer's protocol. Briefly, human embryonic kidney 293FT (HEK293FT) cells were co-transfected with ViraPower Packaging Mix (Thermo Fisher) and a lentivirus vector carrying either the GLUT1 or the Turbo-635 open reading frame using Lipofectamine 2000 transfection reagent. At 24 h after the transfection, the cell culture media were changed to remove the transfection reagent. At 72 h post transfection, the cell culture media containing lentiviral particles were collected. Cell debris were removed by centrifugation, followed by filtration using a 0.45μM PVDF syringe filter unit. The cell culture media containg virus particles were dispensed into 1-mL aliquotes and stored at -80ºC. For the lentiviral transduction, MDA-MB-231 and MRC-5 cell lines were seeded at 40% of the confluency for overnight. Then the cell culture media were replaced with fresh media containg polybrene (2 μg/mL) and virus particles. The cells were allowed to grow for 48 h in the virus-containing media. After that, positively transduced cells were selected using media containing puromycin (2.5 μg/mL, Thermo Fisher Scientific) for at least two weeks. Transduction efficiency was validated by using immunoblot analysis and 2-NBDG uptake assay (Figure 5).

NMR Spectra

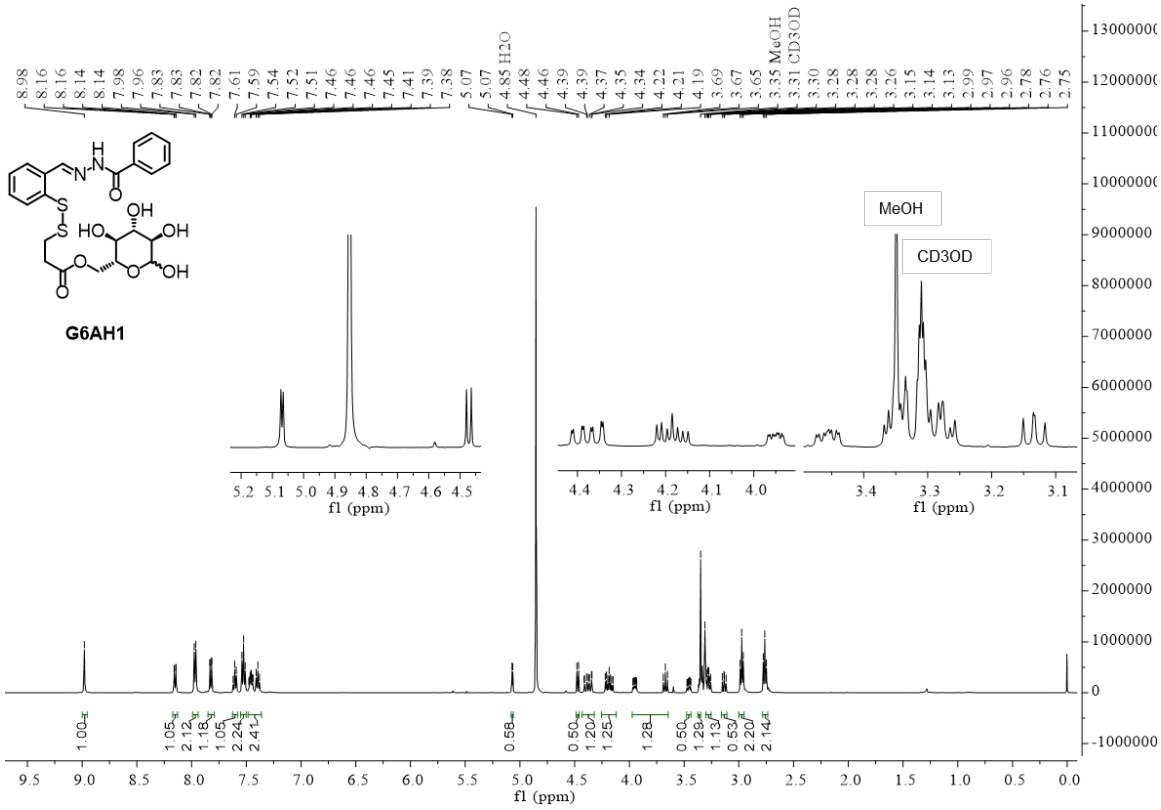


Figure S6. ^{1}H NMR spectrum of **G6AH1** (500 MHz, CD_3OD).

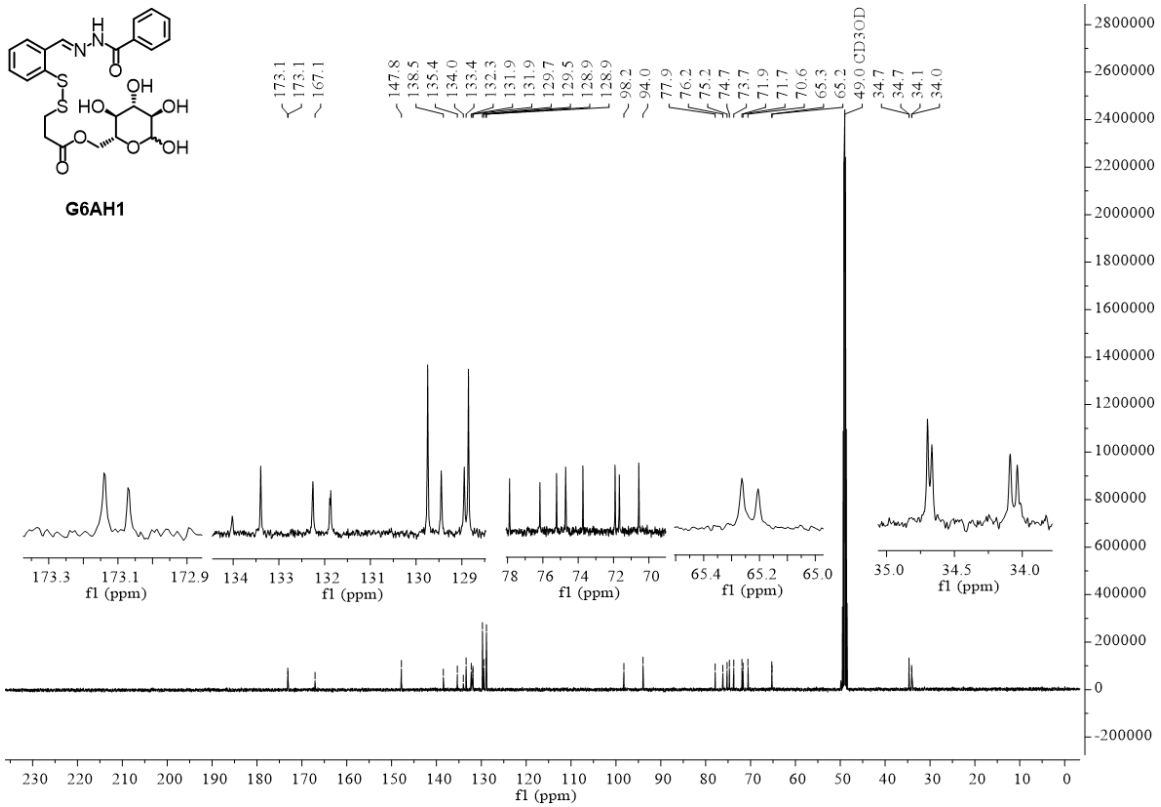


Figure S7. ^{13}C NMR spectrum of **G6AH1** (126 MHz, CD_3OD).

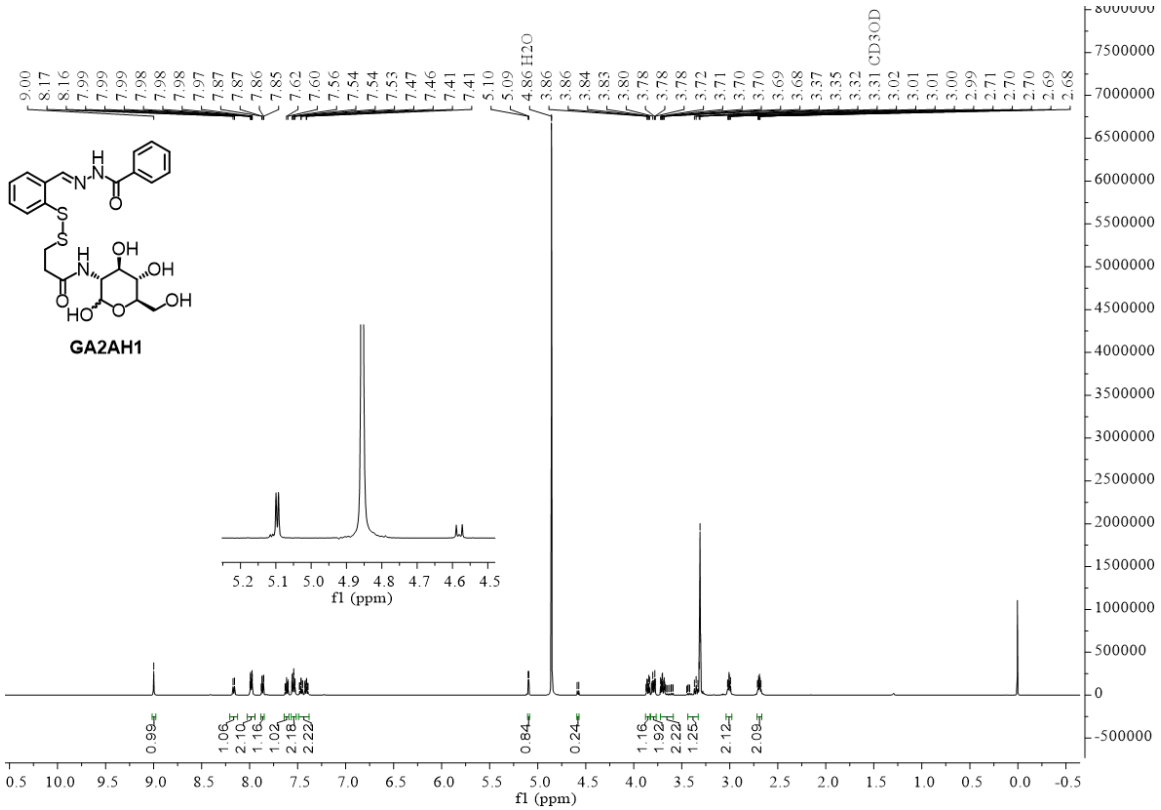


Figure S8. ^{1}H NMR spectrum of **GA2AH1** (500 MHz, CD_3OD).

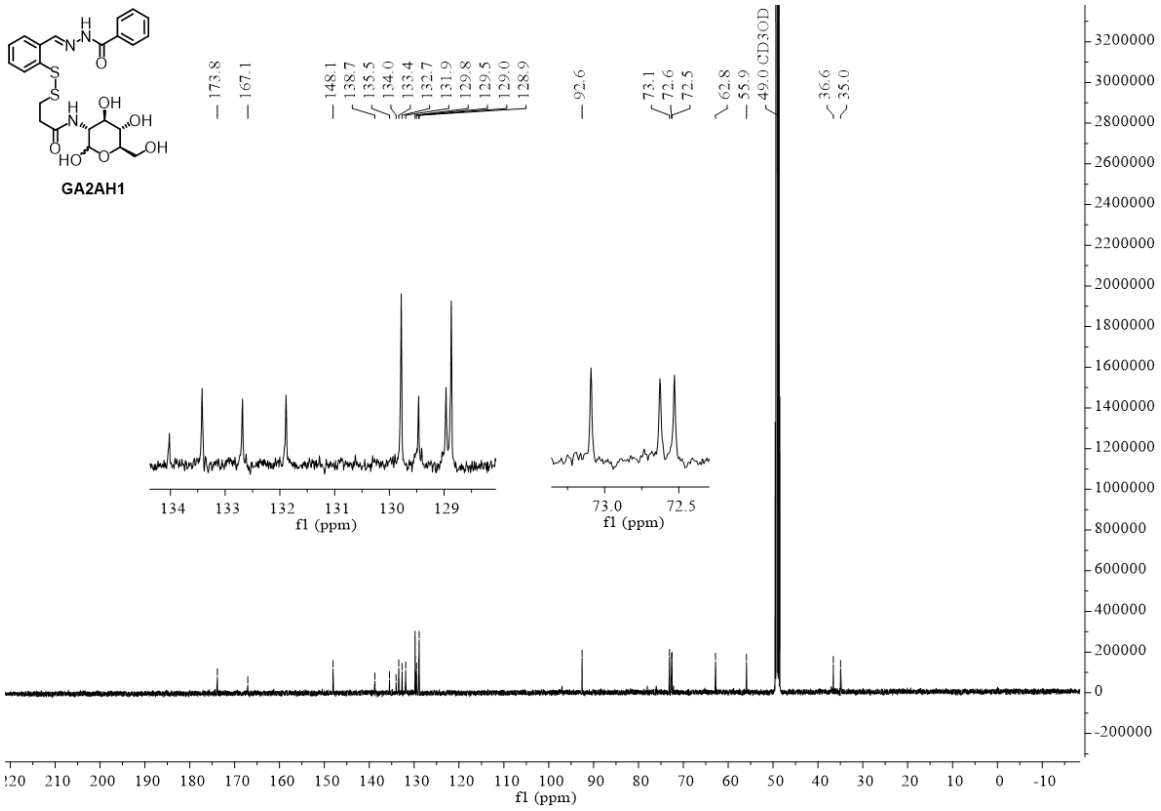


Figure S9. ^{13}C NMR spectrum of **GA2AH1** (126 MHz, CD_3OD).

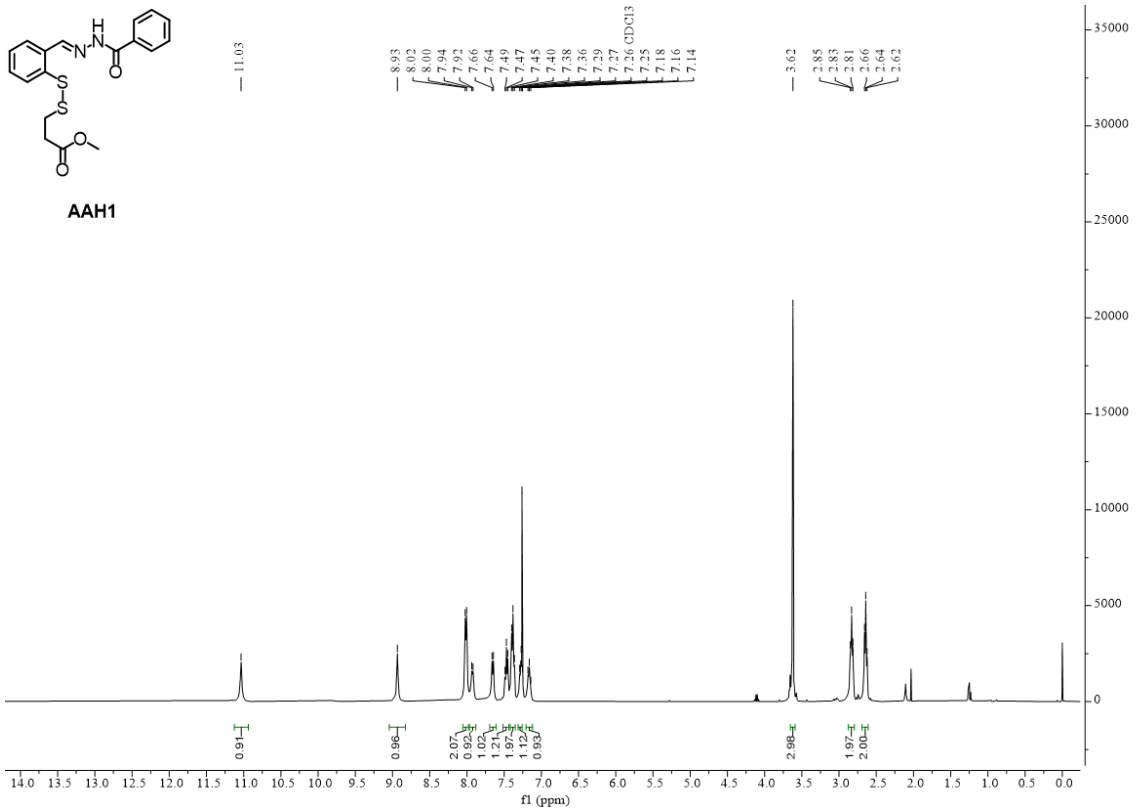


Figure S10. ^{1}H NMR spectrum of **AAH1** (400 MHz, $CDCl_3$).

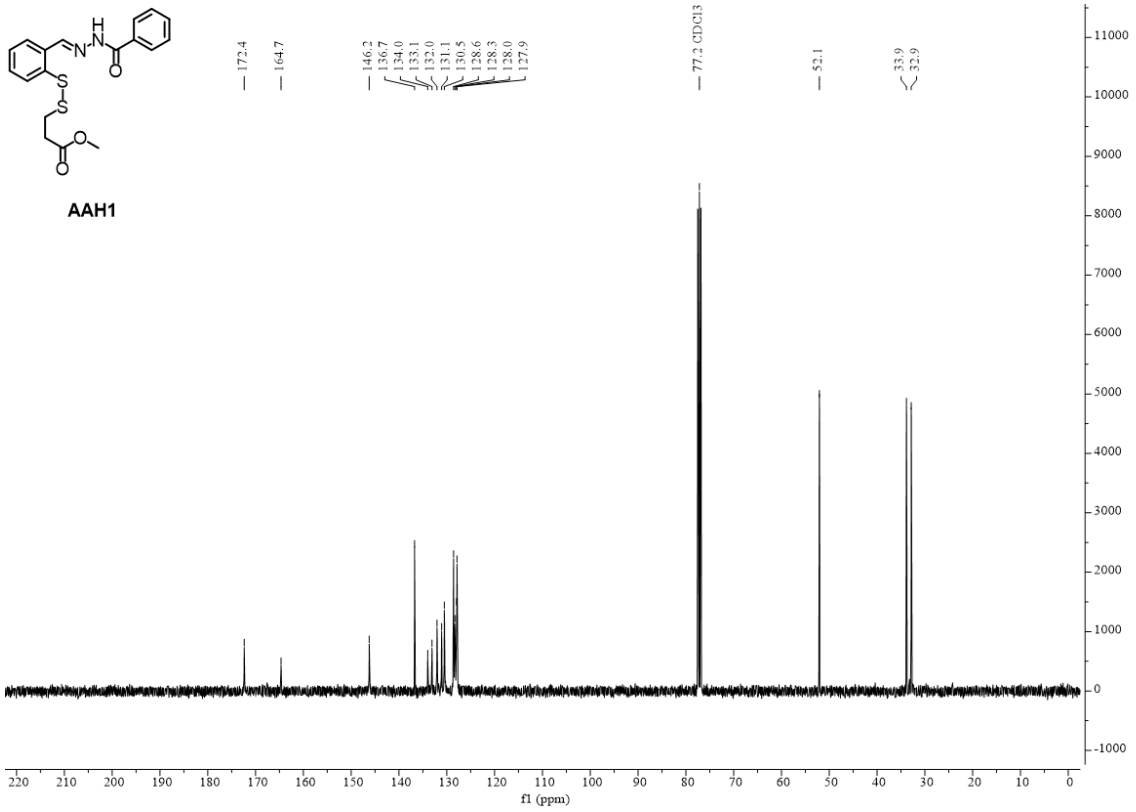


Figure S11. ^{13}C NMR spectrum of **AAH1** (101 MHz, $CDCl_3$).

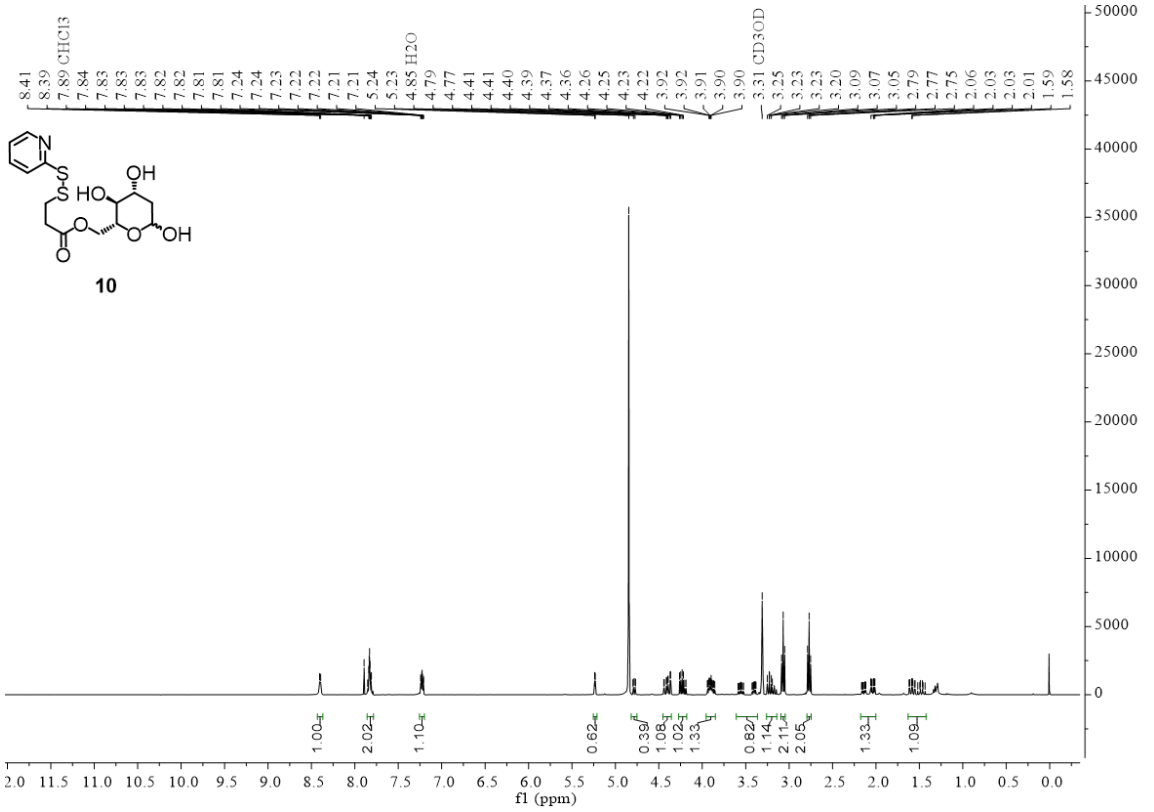


Figure S12. ^{1}H NMR spectrum of **10** (400 MHz, CD_3OD).

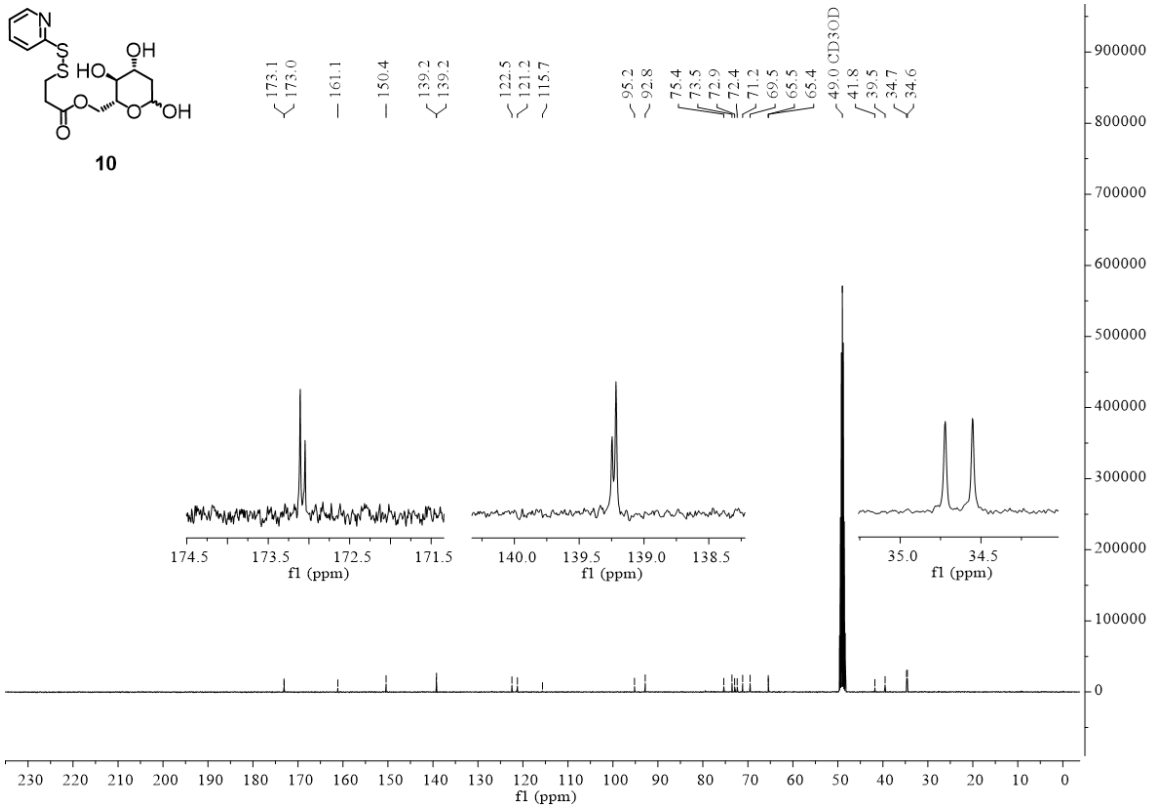


Figure S13. ^{13}C NMR spectrum of **10** (101 MHz, CD_3OD).

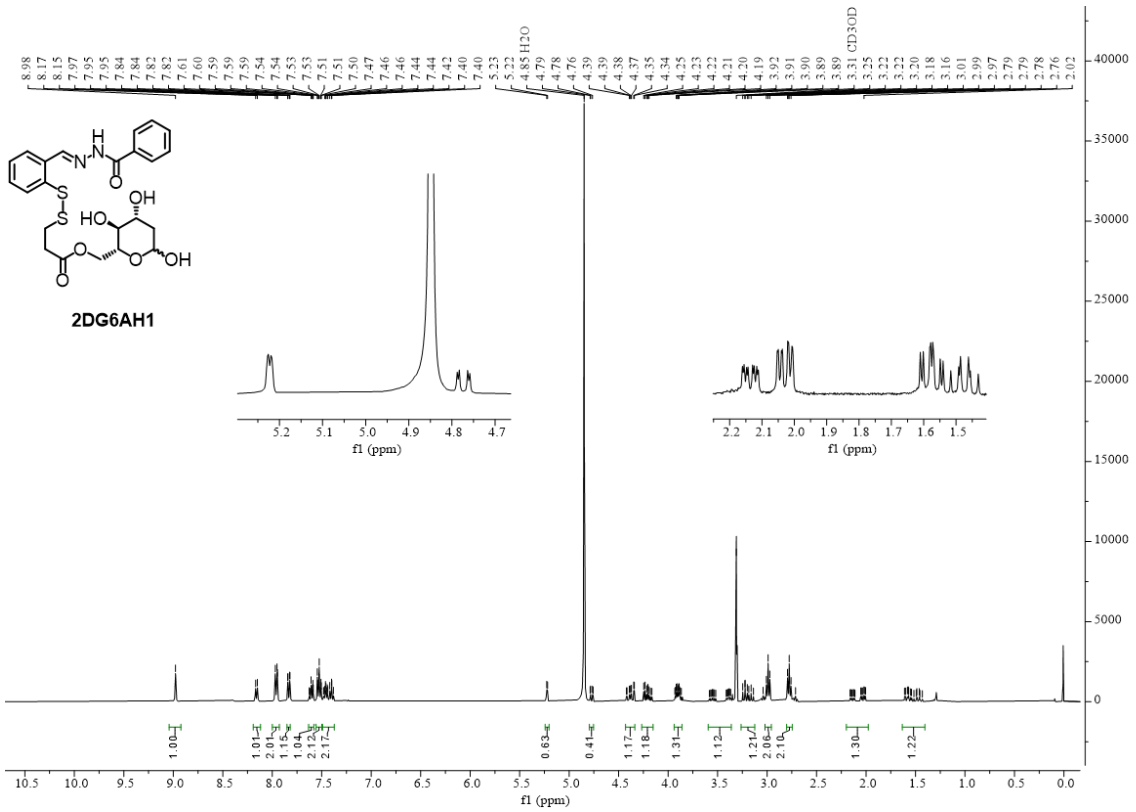


Figure S14. ^{1}H NMR spectrum of **2DG6AH1** (400 MHz, CD_3OD).

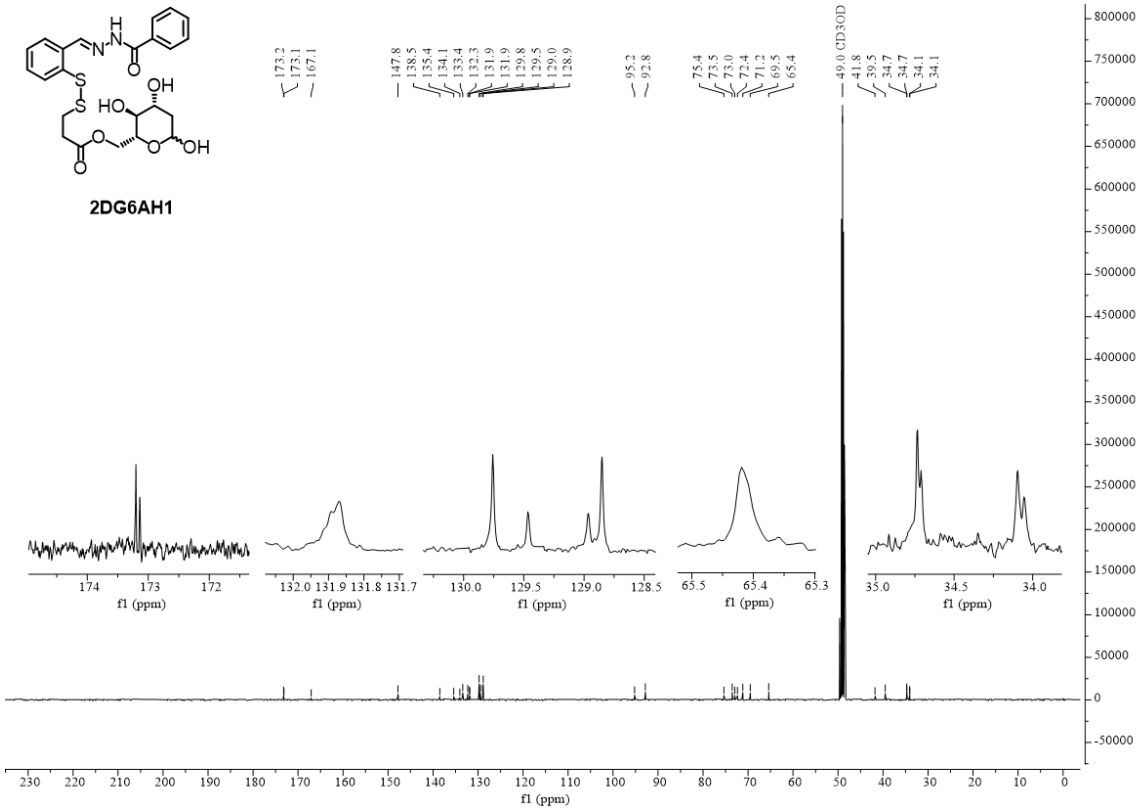


Figure S15. ^{13}C NMR spectrum of **2DG6AH1** (101 MHz, CD_3OD).

References

1. Akam, E. A.; Tomat, E. Targeting Iron in Colon Cancer via Glycoconjugation of Thiosemicarbazone Prochelators. *Bioconjugate Chem.* **2016,** *27,* 1807-1812.
2. Amigues, E. J.; Greenberg, M. L.; Ju, S.; Chen, Y.; Migaud, M. E. Synthesis of cyclophospho-glucoses and glucitols. *Tetrahedron* **2007,** *63,* 10042-10053.
3. Astashkin, A. V.; Utterback, R. D.; Sung, Y.-S.; Tomat, E. Iron Complexes of an Antiproliferative Aroyl Hydrazone: Characterization of Three Protonation States by Electron Paramagnetic Resonance Methods. *Inorg. Chem.* **2020,** *59,* 11377-11384.
4. Michael, E. J. Improved synthesis of 4-amino-7-nitrobenz-2,1,3-oxadiazoles using NBD fluoride (NBD-F). *Tetrahedron Lett.* **2011,** *52,* 2533-2535.
5. Edward, J. T.; Gauthier, M.; Chubb, F. L.; Ponka, P. Synthesis of new acylhydrazones as iron-chelating compounds. *J. Chem. Eng. Data* **1988,** *33,* 538-540.
6. Jones, G. B.; Hynd, G.; Wright, J. M.; Sharma, A. On the Selective Deprotection of Trityl Ethers. *J. Org. Chem.* **2000,** *65,* 263-265.